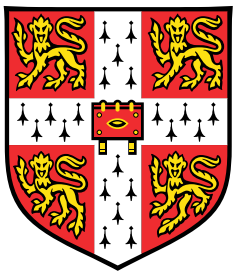
Regulation of oligodendrocyte lineage cell function by the RXRγ nuclear receptor



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To my family and Nick…

Declaration

I hereby declare that except where specific reference is made to the work of others, the contents of this dissertation are original and have not been submitted in whole or in part for consideration for any other degree or qualification in this, or any other university. This dissertation is my own work and contains nothing which is the outcome of work done in collaboration with others, except as specified in the text and Acknowledgements. This dissertation contains fewer than 60,000 words including appendices, bibliography, footnotes, tables and equations and has fewer than 150 figures.

Ludovica Di Canio
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Abstract

Remyelination is a spontaneous regenerative process whereby myelin sheaths are restored to demyelinated axons. Key players in this process are oligodendrocyte progenitor cells (OPCs), a widespread population of CNS progenitor cells which persist into adulthood. Remyelination is impaired in patients with chronic demyelinating conditions such as Multiple Sclerosis, and as with other regenerative processes, its efficiency declines with increasing age. Hence, there is a need for the development of therapeutic interventions that will aid in promoting endogenous remyelination when the endogenous regenerative potential is compromised. The nuclear receptor RXRγ is an important positive regulator of OPC differentiation and an accelerator of endogenous remyelination in aged rats. RXRγ functions as a ligand-induced transcription factor and is able to regulate gene transcription. It does so by heterodimerising with other nuclear receptors and recruiting co-regulators involved in chromatin remodelling. However, we lack understanding on the specific mechanism by which RXRγ promotes OPC differentiation.

With the work presented in this thesis I demonstrate that RXRγ function is regulated at multiple signalling levels. Proximity ligation assays revealed that RXRγ remains consistently bound to its partners throughout the oligodendrocyte lineage, and the biological relevance of each heterodimer is determined by the dynamic association of co-regulators. This is in turn influenced by ligand presence and subcellular receptor localisation. To identify the genes controlled by RXRγ in OPCs I carried out ChIP sequencing, which revealed genes involved in proliferation and cell cycle control. Further functional assessments aided me in the development of a hypothesis whereby RXRγ activation does not directly influence oligodendrocyte formation, but rather promotes cell cycle exit thereby accelerating and facilitating OPC differentiation. Altered nuclear receptor expression and ligand presence in ageing OPCs may consequently impair this process.

My thesis provides an alternative hypothesis to how RXRγ regulates lineage cell progression, highlighting a new avenue in the development of therapeutic interventions targeting generic stem cell functions for which drugs are already FDA approved, rather than oligodendrocyte-specific pathways.

Table of contents

List of figures **xvii**

List of tables **xix**

Nomenclature **xxi**

1 Introduction **1**

1.1 The inadequacy of mammalian brain regeneration 1

1.2 Remyelination is a highly efficient regenerative process in the adult CNS . . 2

1.2.1 The importance of myelin and its regeneration 3

1.2.2 The consequences of myelin loss 6

1.2.3 The stages of remyelination 9

1.3 Oligodendrocyte lineage cells throughout life 14

1.3.1 The role of OPCs in development and adulthood 14

1.3.2 Are OPCs *bona fide* stem cells? 15

1.3.3 Oligodendrogenesis involves transcriptional and morphological changes in OPCs 17

1.4 Ageing leads to remyelination failure 20

1.4.1 The hallmarks of ageing 20

1.4.2 Remyelination efficiency declines with age 21

1.4.3 Multiple Sclerosis is also a disease of age 23

1.4.4 Developing remyelination therapies 24

1.5 RXR belongs to the nuclear receptor superfamily 30

1.5.1 Characterisation of the NR superfamily 30

1.5.2 RXR heterodimers are central to NR signalling 35

1.6 RXR and its partners influence aspects of OPC biology important to remyelination 41

1.6.1 The involvement of RXR and its partners in remyelination 41

1.6.2 RXR and its partners regulate cell cycle and differentiation 43
1.6.3 RXR partners aid in cholesterol and fatty acid metabolism 45
1.7 Aims and objectives 48

2 Materials & Methods **49**
2.1 Materials 49
2.2 Methods 49
2.2.1 Animal husbandry 49
2.2.2 OPC isolation via mixed glia culture 49
2.2.3 OPC isolation via magnetic cell sorting 52
2.2.4 FBS charcoal treatment 54
2.2.5 Small molecule treatment 54
2.2.6 EdU incubation 55
2.2.7 Isolation purity assessment using flow cytometry 55
2.2.8 Immunocytochemistry 56
2.2.9 Co-immuno precipitation 56
2.2.10 Mass spectrometry 58
2.2.11 Proximity ligation assay using Duolink® 58
2.2.12 Cell cycle analysis using flow cytometry 61
2.2.13 OPC dissociation 61
2.2.14 ChIP sequencing 61
2.2.15 Concentrating DNA 63
2.2.16 Western blot 63
2.2.17 RNA extraction and purification 65
2.2.18 cDNA synthesis 65
2.2.19 Quantitative PCR 66
2.2.20 Statistical analysis 68

3 Results **69**
3.1 Mass spectrometry analysis of RXR complexes in OPCs 69
3.1.1 Purity and viability of acutely isolated OPCs 70
3.1.2 RXR CoIP and mass spectrometry analysis 72
3.1.3 Troubleshooting the mass spectrometry analysis of RXR complexes 74
3.1.4 Discussion 76
3.2 Levels of control for RXR-NR signalling in oligodendrocyte lineage cells . 79
3.2.1 RXR activation in serum-free conditions 79
3.2.2 Purity of cultured OPCs isolated via MACS 80

3.2.3 RXR activation alone is not sufficient to promote OPC differentiation 84
3.2.4 RXR is constantly bound to its partners during OPC differentiation 92
3.2.5 The subcellular location of RXR heterodimers during OPC differentiation . . . 98
3.2.6 Co-regulator association to RXR partners during OPC differentiation 102
3.2.7 Discussion . . . 106
3.3 Identifying the genes controlled by RXR in oligodendrocyte lineage cells: a ChIP-seq approach . . . 109
3.3.1 Genome-wide profiling of RXR in primary rat OPCs . . . 113
3.3.2 RXR HREs are located in proximity of genes involved in regulation of proliferation and stem cell control . . . 115
3.3.3 Discussion . . . 117
3.4 RXR activation regulates OPC proliferation . . . 121
3.4.1 9cRA treatment decreases OPC proliferation in serum-free conditions 122
3.4.2 Testing the effect of RXR on the cell cycle and the exit marker p27 126
3.4.3 Discussion . . . 131
3.5 Nuclear receptors in ageing OPCs . . . 135
3.5.1 NR expression in ageing OPCs . . . 136
3.5.2 Discussion . . . 137

4 Final discussion 141
4.1 RXR activation facilitates differentiation by influencing OPC proliferation: a new hypothesis . . . 141
4.2 RXR activation and G1 lengthening in the ageing OPC . . . 145
4.3 Is targeting RXR and its partners a valid strategy for remyelination therapies in MS? . . . 147
4.4 The complexity of NR signalling and clinical trial design . . . 149
4.5 Directions for future research . . . 151

5 Concluding remarks 155

References 159

Appendix A 203

List of figures

1.1 The consequences of primary demyelination 8
1.2 The stages of remyelination 12
1.3 The oligodendrocyte lineage differentiation timeline 19
1.4 Factors affecting OPC differentiation 26
1.5 Schematic of NR structure 33
1.6 The signalling mechanism of RXR heterodimers 34
1.7 Levels of control for NR signalling 36
1.8 Partner switching model 47

2.1 Mixed glia isolation 50
2.2 Magnetic cell sorting 54
2.3 Duolink® *in situ* proximity ligation assay technology 60

3.1 Purity of acutely isolated OPCs 71
3.2 Mass spectrometry analysis of RXRγ CoIP 73
3.3 Mass spectrometry troubleshooting 75
3.4 Purity of primary rat OPCs extracted by MACS and cultured in serum free conditions 83
3.5 Titrations assessing cell death of NR ligand treatments 87
3.6 NR ligand treatment in serum-free conditions 89
3.7 Spontaneous OPC differentiation after normal serum, charcoal-treated serum and serum-free media exposure 91
3.8 Experimental set up for Duolink® assay 95
3.9 RXRγ does not switch partners during OPC differentiation 97
3.10 Nucleocytoplasmic shuttling of RXRγ heterodimers over the course of OPC differentiation. 101
3.11 Co-regulator association to RXRγ partners is more dynamic 105

3.12 Summary of the observations made on the levels of control for RXRγ-NR signalling in oligodendrocyte lineage cells 108
3.13 Experimental design and controls for RXRγ ChIP-seq in primary rat OPCs 110
3.14 Purity of OPC cultures prior to ChIP-seq 111
3.15 Genome-wide RXRγ binding in the absence and presence of 9cRA 114
3.16 RXRγ mainly associates to genes unique to OPCs and newly formed oligodendrocytes 116
3.17 Effect of 9cRA on the proliferation of MACS sorted rat primary OPCs cultured in serum-free conditions 124
3.18 Immunocytochemistry of 9cRA effect on OPC proliferation and morphology 125
3.19 Changes in cell cycle markers upon RXR activation 129
3.20 OPC distribution across cell cycle stages upon RXR activation 130
3.21 Changes in NR protein and RNA levels in ageing OPCs 139

4.1 RXR activation facilitates differentiation by influencing OPC proliferation: a new model 144

List of tables

1.1 The nuclear receptor superfamily 32

2.1 Formulation for HALF 51
2.2 Formulation for OPC media 52
2.3 Formulation for MWB 53
2.4 Antibodies used to assess purity of the MACS isolation using flow cytometry 55
2.5 Antibodies used for *in vitro* experiments 57
2.6 Antibodies used for *in vitro* proximity ligation assay using Duolink® 59
2.7 Antibodies used for western blot experiments 64
2.8 Set up for qPCR 66
2.9 Parameters for qPCR run 66
2.10 Forward and reverse primer sequences for the qPCR targets selected 67

3.1 Co-regulators involved in myelin cell biology 102

A.1 Materials 205

Nomenclature

Roman Symbols

9cRA 9-*cis*-retinoic acid

ALDH1A1 Aldehyde dehydrogenase 1A1

ANOVA Analysis of variance

APL Acute promyelocytic leukaemia

ATAC-seq Assay for transposase-accessible chromatin using sequencing

ATP Adenosine triphosphate

ATRA All-*trans* retinoic acid

BMP Bone morphogenic protein

CC Corpus callosum

CDK Cyclin dependent kinase

cDNA complementary DNA

ChIP Chromatin immunoprecipitation

CNPase 2',3'-cyclic nucleotide 3'-phosphodiesterase

CNS Central nervous system

CoIP Co-immunoprecipitation

COUP-TF Chicken ovalbumin upstream promoter transcription factor

CSPG Chondroitin sulfate proteoglycans

CYP Cytochrome P450

DBD DNA binding domain

ddH_2O double distilled water

DSS 4,4-dimethyl-4-silapentane-1-sulfonic acid

E Embryonic day

EAE Experimental autoimmune encephalomyelitis

EB Ethidium bromide

ECM Extracellular matrix

EdU Ethynyl-2'deoxyuridine

EM Electron microscopy

ENPP6 Ectonucleotide pyrophosphatase/phosphodiesterase family member 6

ER Oestrogen receptor

ESC Embryonic stem cell

FBS Fetal bovine serum

FGF Fibroblast growth factor

FMO Fluorescence minus one

FRET Fluorescence resonance energy transfer microscopy

FUCCI Fluorescence ubiquitination-based cell cycle indicator

FXR Farnesoid X receptor

GCR Glucocorticoid receptor

GFs Growth factors

GO Gene ontology

GSK3 Glycogen synthase kinase 3

H3K27ac Histone H3 acetyl Lys27

HALF Hibernate A for low fluorescence

HBSS Hank's balanced salt solution

HDAC Histone deacetylase

HP1α Heterochromatin protein 1α

HRE Hormone response element

HSC Hematopoietic stem cell

HX HX531

iPSC Induced pluripotent stem cell

kD Kilodalton

LBD Ligand binding domain

LINGO1 Leucine-rich repeat and immunoglobulin-like domain-containing nogo receptor-interacting protein 1

LXR Liver X receptor

mAchR Muscarinic acetylcholine receptor

MACS Magnetic cell sorting

MBP Myelin basic protein

MCT Monocarboxylate transporter

MEME Multiple EM for motif elicitation

mESC Mouse embryonic stem cell

MOBP Myelin-associated oligodendrocyte basic protein

MS Multiple Sclerosis

MSC Mesenchymal stem cell

Mw Molecular weight

MWB Miltenyi Wash Buffer

Myrf Myelin regulatory protein

NcOR Nuclear co-repressor

NICD Notch intracellular domain

NLS Nuclear localisation sequence

NMR Nuclear magnetic resonance spectroscopy

NR Nuclear receptor

NSC Neural stem cell

OLG Oligodendrocyte

OPC Oligodendrocyte progenitor cell

P Postnatal day

PDGF Platelet-derived growth factor

PDL Poly-D-Lysine-hydrobromide

PFA Paraformaldehyde

PI Propidium iodide

PLP Proteolipid protein

PMD Pelizaeus-Merzbacher disease

PNS Peripheral nervous system

PPAR Peroxisome proliferator activated receptor

PPMS Primary progressive MS

PSC Pluripotent stem cell

PXR Pregnane X receptor

R point Restriction point

RA Retinoic acid

RAR Retinoic acid receptor

RRMS Relapsing remitting MS

rRXRγ Recombinant RXRγ

RT Room temperature

RXR Retinoid X receptor

SD Sprague Dawley

SMRT Silencing mediator for retinoic and thyroid hormone receptor

SPMS Secondary progressive MS

SREBP1c Sterol regulatory element-binding protein 1c

T090 T0901317

T3 Triiodothyronine

TGF Transforming growth factor

THR Thyroid hormone receptor

TLR Toll-like receptor

TSS Transcription start site

TTDS Tetraethylthiuram disulfide

VDR Vitamin D receptor

ZV Zombie violet

Chapter 1

Introduction

1.1 The inadequacy of mammalian brain regeneration

Regeneration is the ability of an organism to replace damaged tissues and organs. Regenerative processes can be divided into two types: physiological and reparative. The former consists of the spontaneous replacement of worn out tissue to meet physiological needs in order to preserve homeostasis. Examples are the continuous replacement of the skin as well as the great production of red blood cells at changing altitudes to meet oxygen demands (Sawka et al., 2000; Chuong et al., 2012). Instead, reparative regeneration occurs post-traumatically and is able to replace whole damaged tissue parts.

All organisms have the capability to regenerate. However, the extent to which they can do so varies greatly across phyla and does not necessarily present a phylogenic relationship (Brockes and Kumar, 2008). Invertebrates are amongst the most fascinating examples of organisms with great regenerative capabilities. The freshwater *Hydra* polyp was the first organism to be studied in the context of biological regeneration. It has been observed that both small fragments as well as single cells of *Hydra* can completely regenerate a whole new animal (Gierer et al., 1972; Glass, 1988). Some vertebrates are also capable of dramatic regeneration. The urodele amphibians (newts and axolotls) are an exceptional example as they are the only adult vertebrates capable of regenerating whole limbs, tail, jaw and much more.

Conversely, mammals do not present such outstanding regenerative capacity. Due to the discrepancies in regenerative capability across the animal kingdom, the importance of studying regeneration was recognised from the very first studies in the field (Morgan, 1901), and today regenerative medicine presents an important clinical challenge for the scientific community. In adult mammals we observe differences in the regenerative capacity of various organs and tissues within the same organism. Regenerative ability is directly related

to the presence of progenitor cells in the organs of interest (Iismaa et al., 2018). Tissues with successful regeneration tend to have a niche of highly proliferative stem cells including, for example, the skin or the intestine. In contrast, systems such as the central nervous system (CNS) tend to present poor regenerative capacity. Once mature neurons are formed throughout embryonic development, neural stem cell (NSC) numbers deplete and neurogenesis no longer occurs, with the exception of two brain areas: the granular layer in the dentate gyrus of the hippocampus and the sub-ventricular zone of the lateral ventricles (Kaplan and Hinds, 1977; Lois and Alvarez-Buylla, 1993; Eriksson et al., 1998; Merkle et al., 2004; van Wijngaarden and Franklin, 2013). In contrast to the deficient regenerative quality of adult neurogenesis after the loss of neurons, loss of oligodendrocytes leads to a highly successful CNS regenerative process termed remyelination (Franklin and ffrench Constant, 2017).

1.2 Remyelination is a highly efficient regenerative process in the adult CNS

Oligodendrocytes are the glial cells responsible for the myelination of neuronal axons in the CNS. Myelin is essential to neuronal function and survival as it acts as an insulating layer allowing for fast and efficient propagation of action potentials, as well as providing trophic support to the axon. In young mammals, primary demyelination is followed by the robust process of remyelination, whereby whole myelin sheaths can be restored along the denuded axon (Franklin and ffrench Constant, 2017). This has been elegantly shown with numerous toxin-induced models of primary demyelination. These experimental models commonly use toxins such as injection of ethidium bromide (EB) or lysolecithin in white matter tracts, or oral administration of cuprizone to specifically kill oligodendrocytes (Blakemore and Franklin, 2008). The common goal is to induce primary demyelination, however, their mechanism of action differs. EB is injected in either the rat caudal cerebellar peduncle or the mouse spinal cord. Being an intercalating agent, EB kills all nucleated cells in the area, thereby sparing neurons but not glial cells when injected into the white matter (Blakemore, 1982, 2005). Lysolecithin is a membrane solubilising agent to which oligodendrocytes are specifically susceptible (Hall, 1972; Jeffery and Blakemore, 1995). This makes lysolecithin a more delicate toxin compared to EB, as usually astrocytes, microglia and other glial cells tend to be relatively spared. Lysolecithin is typically injected into the corpus callosum (CC) or into the spinal cord. Copper chelator cuprizone is less invasive as it is administered orally alongside the standard animal diet (Blakemore, 1972, 1973). It leads to global demyelination, but the prominent area affected is the CC (Matsushima and

Morell, 2001). Demyelination is caused by the dysfunction of the mitochondrial complex IV to which oligodendrocytes are vulnerable (Venturini, 1973; Faizi et al., 2016). In these models (although to a lesser extent for cuprizone) the lesion is focal, thereby making it defined and easy to find and follow over time. Additionally, these models do not have an adaptive immune mediated demyelination component, therefore demyelination is spatiotemporally separated from the remyelination phase, allowing the study of the regenerative process *per se*.

Remyelination is also observed in Multiple Sclerosis (MS). MS is an autoimmune primary demyelinating disease and is the prominent cause of neurological disabilities among the young population. Despite the myelin restoration, ultimately remyelination fails causing an accumulation of disability (Patrikios et al., 2006; Goldschmidt et al., 2009). MS, alongside leukodystrophies, which are demyelinating diseases due to genetic defects in myelin related genes, are clinical examples of how crucial myelin is to our basic function and survival (Franklin and ffrench Constant, 2017).

1.2.1 The importance of myelin and its regeneration

Myelin is produced and maintained by oligodendrocytes in the CNS and Schwann cells in the peripheral nervous system (PNS). It is formed by an outgrowth of the membrane of these cells, which wraps around the axon multiple times and finally is compacted. In contrast to conventional cell membranes, myelin presents a substantially higher dry mass of lipids (70-85%), the prevalent one being cholesterol, and is composed to a lesser extent of proteins (15-30%) involved in its compaction, substance transport and exchange (Baumann and Pham-Dinh, 2001; Nave and Trapp, 2008). Myelin allows for faster action potential conduction (Waxman and Bennett, 1972). This is achieved by myelin acting as an insulator, decreasing the capacitance and increasing the membrane resistance of axons. This confers an evolutionary advantage, as the only other way to achieve the same effect in their unmyelinated counterparts is by dramatically enlarging the axon diameter (Gillespie and Stein, 1983; Zalc et al., 2008). This is because the action potential propagation speed and axon diameter are directly proportional in unmyelinated axons. Only cephalopods such as the squid have been able to develop 'giant axons', which are mainly involved in their escape response. Vertebrates were unable to do the same as it implied an impractical substantial increase in the size of the nervous system (Zalc et al., 2008). Instead, vertebrate myelin evolved in the jawless fishes for the development of the aforementioned fast escape reflexes, and approximately 600 million years later we find naturally selected myelinated preys and predators (Nave and Trapp, 2008). Myelin, therefore, allowed for higher computational power, complexity and faster information transmission within a reasonably sized nervous system. The myelinated

areas along axons are termed internodes, in which the insulating property of myelin resides. Due to its compaction, myelin acts as a barrier preventing ion diffusion. Consequently, the only area where ion diffusion and exchange can occur is in the non-myelinated paranodal areas called 'nodes of Ranvier'. Here we find clustering of ion channels and transporters such as voltage-gated Na^+ channels, K^+ channels and Na^+-K^+ pumps (Mata et al., 1991).

Voltage-gated Na^+ channels allow for Na^+ entry and propagation of the action potential after axonal membrane depolarisation, and their intervallic clustering at the nodes of Ranvier forms the basis for 'saltatory conduction', which is able to speed action potential conduction 10 times more than in their unmyelinated counterparts (Salami et al., 2003). The Na^+-K^+ pumps are involved in maintaining the resting potential by pumping Na^+ out of the axon and K^+ into it, and are ATP driven as they need to pump both these ions against their concentration gradients. In myelinated axons the Na^+-K^+ pumps are also restricted to the nodes of Ranvier.

As the area over which they are distributed is reduced, less energy is required to restore and maintain the membrane potential following a depolarisation event (Harris and Attwell, 2012). Therefore, not only does myelin promote fast action potential propagation, but it also allows the neuron to restore membrane potential in a less metabolically demanding condition. However, calculations by Harris and Attwell (2012) suggest that, when considering the energy required to produce and maintain oligodendrocytes and myelin, myelination does not result in a reduction in energy consumption. The importance of myelin compaction for correct action potential transmission is apparent when observing the homozygous autosomal recessive mutant *shiverer*, a transgenic mouse lacking myelin basic protein (MBP). MBP is a structural protein essential to the compaction of myelin and its absence in the *shiverer* mouse leads to severe myelin decompaction, resulting in a distinct phenotype involving tremors around the onset of developmental myelination, followed by progressively worse tonic seizures as the mouse develops and resulting in premature death 90-150 days after birth (Readhead, 1987). Due to impaired myelin compaction, *shiverer* mice may lack defined internodes. This is supported by the dispersed distribution of K^+ channel subunits along the dysmyelinated axons of these mice (Sinha et al., 2006; Eftekharpour et al., 2007). The same dispersion may also occur for Na^+ channels, thereby compromising the highly structured organisation of the nodes, essential for action potential propagation and recovery. However, this mutant does not present neurodegeneration, suggesting an inability to correctly control action potential propagation and signal integration at the neural network level.

A more recently described function of myelin is that of metabolically supporting the axon, thereby promoting both its function and survival. Neurons are metabolically expen-

sive cells due to their highly specialised design and function. With axons projecting as far as the length of the whole spinal cord, neurons require an efficient transport system from the soma to the axon in order to transport organelles, newly synthesised proteins and energy metabolites necessary to avoid neurodegeneration. Energy to support this transport system is likely produced throughout the length of the axon where glucose transporters are located. However, whilst unmyelinated neurons are accessible to energy metabolites present in the extracellular space throughout their entire axon, in myelinated neurons the area directly exposed to the extracellular space is limited to the unmyelinated Nodes of Ranvier (Morrison et al., 2013). The presence of myelin makes axons inaccessible for most of their surface area, thereby preventing the direct transport and diffusion of energy metabolites such as glucose (Mierzwa et al., 2010). It has therefore been hypothesised that metabolic substrates must be provided by the oligodendrocyte and transported into the axon via the myelin (Morrison et al., 2013). Evidence of this comes from both transgenic animals lacking myelin specific proteins as well as clinical cases. Pelizaeus-Merzbacher disease (PMD) is a leukodystrophy presented by patients with a mutation in the gene encoding for proteolipid protein (PLP) 1, and the first observations of oligodendrocyte metabolic support derived from the study of *PLP1 null* mice. These mice develop and myelinate normally, however, after 12 months they present axonal swelling, axon loss and neurodegeneration (Griffiths et al., 1998). Although the exact mechanism leading to axon degeneration in these mice is still unknown, it has been hypothesised that it could be due to impaired axonal transport (Edgar et al., 2010). Another oligodendrocyte specific gene that has been linked to metabolic support of axons is *CNP1*, which encodes for the enzyme 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase). *CNP1 null* mice also show severe neurodegeneration, however by electron microscopy (EM) they do not present defects in myelin compaction, nor demyelination (Lappe-Siefke et al., 2003; Rasband et al., 2005). Using high-pressure freezing EM, Snaidero et al. (2014) visualised a system of cytoplasmic channels between the compacted layers of developing myelin. The channels run from the oligodendrocyte cell body to the inner most myelin tongue which is directly in contact with the axon. Within these channels, it is possible to observe microtubules and vesicles, components essential for motor-driven transport. The group further observed that the cytoplasmic channels are kept throughout adulthood and identified CNPase to be essential in the maintenance of such channels (Snaidero et al., 2017). Mechanistically, CNPase antagonises the membrane compaction exerted by MBP at specific locations, thereby creating pockets of cytoplasm between the oligodendrocyte cell body and the axon. It is thought that these spaces allow for efficient diffusion of metabolites and other cargo (Snaidero et al., 2017). Therefore, it can be speculated that CNPase-deficient mice lack cytoplasmic channels due to complete compaction of myelin, consequently impairing

metabolite diffusion and transport to the axon and compromising its function and survival. Further evidence of the crucial role of oligodendrocytes in metabolically supporting axons comes from their involvement in lactate transport to the neurons via the extracellular membrane transport channel MCT1. Monocarboxylate transporters (MCT) are transporters involved in the transport of pyruvate, lactate, ketone bodies and so on. In the CNS, MCT1 is found predominantly on oligodendrocytes and MCT2 is localised on neurons. Based on their distribution, a model for intercellular shuttling of lactate for energy transfer had already been hypothesised in the nineties by Pellerin et al. (1998). However, the group attributed the supply of lactate largely to astrocytes. It was not until MCT1 was experimentally downregulated or pharmacologically blocked specifically in oligodendrocytes that its importance to neuronal survival became apparent. MCT1 inhibition via antisense oligonucleotides or drugs in organotypic spinal cord cultures leads to axon injury and neuronal loss of motor neurons (Lee et al., 2012). This is especially true when they are cultured in glucose-free media, as under these conditions neurons become dependent on lactate: preventing oligodendrocyte-dependent lactate release results in neuronal death without affecting the oligodendrocyte. Neuronal death can be rescued by adding lactate directly into the cultures. *In vivo*, downregulation of MCT1 via lentiviral delivery of shRNA and oligodendrocyte-specific MCT1 knock out mice both present axon degeneration and in some cases neuronal loss (Lee et al., 2012; Fünfschilling et al., 2012). Despite the lactate flux from the oligodendrocyte to the neuron is never measured directly in these studies, they still make a strong case for the importance of oligodendrocyte lactate transporters for neurons. It has therefore been hypothesised that MCT transporters in the myelin may provide axonal mitochondria with lactate, glucose and other metabolites to produce enough energy for axonal maintenance, and their disruption can lead to neurodegeneration (Lee et al., 2012; Fünfschilling et al., 2012).

1.2.2 The consequences of myelin loss

Primary demyelination is a pathological process characterised by the loss of myelin sheaths wrapping axons. Unlike in Wallerian degeneration, where myelin degenerates as a consequence of axonal loss, primary demyelination results from an insult directly targeting the oligodendrocyte, subsequently leading to its death (Franklin and ffrench Constant, 2008). Demyelination can occur as a result of numerous insults of different origin such as genetic, in the case of leukodystrophies, inflammatory, viral, hypoxic-ischaemic or due to acquired metabolic defects (Love, 2006). Regardless of its causes, however, demyelination results in a conduction block leading to impaired function (Smith et al., 1979).

The default response to all demyelinating insults is remyelination, which is able to restore myelin sheaths around denuded axons and resolve the conduction block (Figure 1.1).

Evidence for this comes not only from toxin-demyelinated animal models, but also from pathological studies of post mortem tissues of MS patients, where the incidence and distribution of remyelinated lesions has been analysed. Studying heterogeneous patients with varying clinical courses and durations of the disease, as well as the ample sampling of lesions from only two patients with a long clinical course, revealed extensive remyelination occurring in MS lesions (Patrikios et al., 2006; Patani et al., 2007; Boyd et al., 2013). Across the global population of MS patients, 20% present highly remyelinated shadow-plaques, a percentage which appears to be independent of relapsing-remitting or progressive MS (Patrikios et al., 2006). One of the limitations of these studies is the use of myelin-specific dyes such as Luxol fast blue to identify shadow-plaques when *bona fide* remyelination can only be identified using EM.

Remyelinated fibres are identified by the correlation between the axon diameter and the myelin sheath thickness. This relationship is called the *g* ratio and is calculated by dividing the axon diameter by the diameter of the axon and myelin. Developmental myelination presents a relationship between axon diameter and myelin thickness, where the larger the axon diameter, the thicker the myelin wrapping it (Hildebrand and Hahn, 1978). However, using the toxin-induced demyelination animal models, it has been clearly shown that the *g* ratio is never restored to the original value obtained in development. This appears to be independent of the time the animals are left to remyelinate for, and even after six months of recovery, original *g* ratio values are not reached in large diameter axons (Blakemore, 1974; Ludwin and Maitland, 1984). The above relationship is less clear in smaller diameter axons as it is hard to distinguish the *g* ratio of remyelinated axons from that of normally myelinated axons (Stidworthy et al., 2003). Therefore, the only way to assert that remyelination has truly occurred is by carrying out EM and calculating the *g* ratio of the lesion of interest.

Electrophysiological studies have demonstrated that despite the abnormally thin myelin, remyelination restores conduction in the previously demyelinated axons. Electrodes implanted in the spinal cord of cats were used to record neuronal action potentials in the demyelinated lesion itself, two months prior to lysolecithin injection, and up to four months after lesion induction (Smith et al., 1979). Recordings showed that in demyelinated lesions activity is almost nil, with small, very weak peaks of activity being recorded. This could be due to the reported observation that upon demyelination axons redistribute Na^+ channels along the denuded axons to try and preserve some form of conduction (Felts et al., 1997). Weak conduction is regained at approximately the onset of remyelination and sheath thickening over time correlates with the activity returning to the levels recorded prior to demyelination (Smith et al., 1979). This suggests that remyelination safely restores saltatory conduction, which in turn translates into functional recovery (Liebetanz and Merkler, 2006;

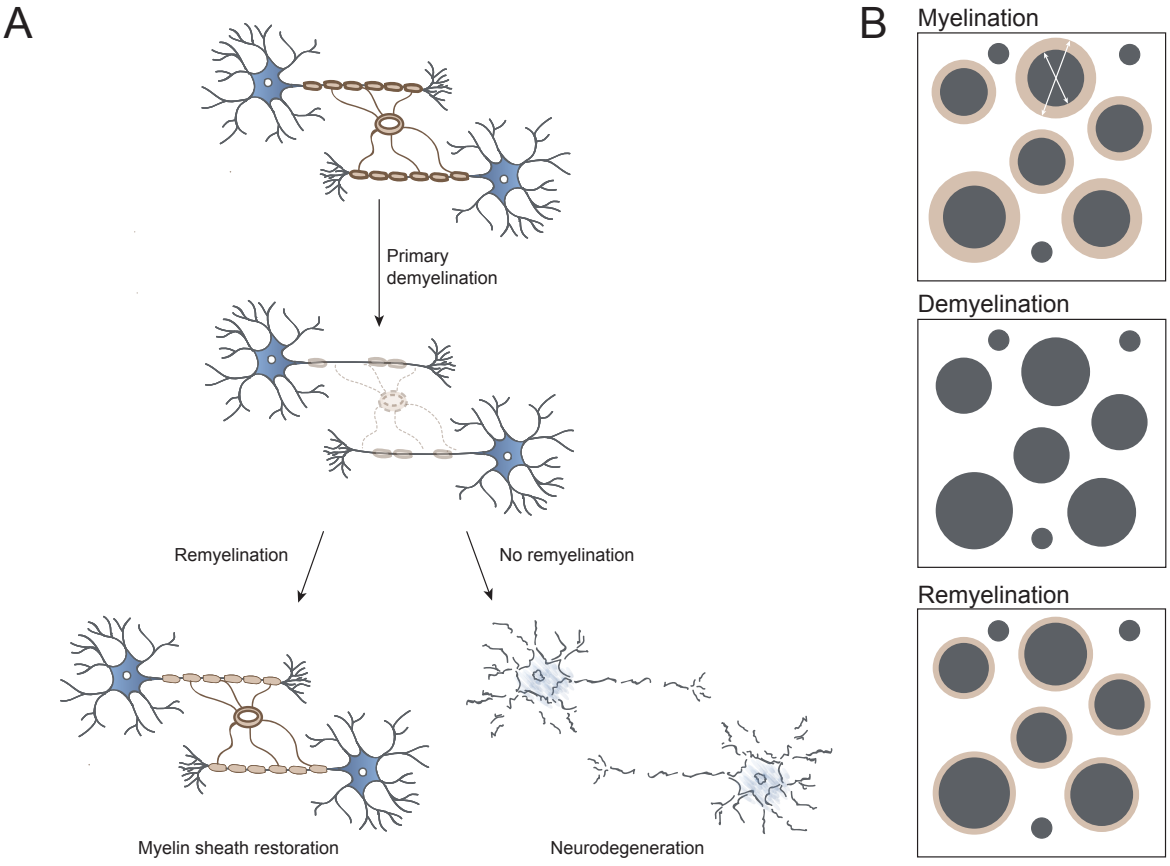


Fig. 1.1 The consequences of primary demyelination. (A) Oligodendrocytes wrap myelin around axons; when oligodendrocytes die as a consequence of an insult, the neurons are demyelinated leading to conduction block. The default response to demyelination in a young CNS is the generation of new myelin sheaths and remyelination. If remyelination is impaired, as in the case of MS, neurons are left vulnerable to degeneration resulting in functional deficits. (B) A schematic of a white matter transverse section: the top panel shows axons myelinated by developmental myelin and the criteria used to calculate the *g* ratio. The middle panel represents demyelinated axons, a process which can be induced experimentally by focal injection of toxins such as EB and lysolecithin. Following demyelination induction, we can observed remyelinated axons presenting thinner myelin sheaths than the original developmental myelin.

Duncan et al., 2009). Due to this, remyelination is thought to be the cause of the remission phases in relapsing-remitting MS (Compston and Coles, 2008).

A lower incidence of neurodegeneration has been observed in remyelinated lesions compared to chronically demyelinated ones, in both human MS lesions and in experimental autoimmune encephalomyelitis (EAE), the most widely used animal model for MS (Kornek et al., 2000). Following from the axonal trophic support provided by myelin, it is logical that remyelination could aid in preventing neurodegeneration in the setting of chronic demyelinating diseases. However, the conclusions made by Kornek et al. (2000), as well as in other similar studies, are simply observational and therefore describe correlation without demonstrating causality. More functional approaches have involved the prevention of remyelination by X-irradiating cuprizone treated mice (Irvine and Blakemore, 2008). These animals show increased neurodegeneration compared to the non-irradiated controls. This phenotype could be rescued by transplanting neural progenitors which differentiated into oligodendrocyte lineage cells *in vivo*. Despite these studies present strong evidence for the neuroprotective role of remyelination, we still cannot determine whether remyelination is the cause of increased neuronal survival in a disease setting or whether remyelination occurs in areas where axons are alive and have the potential to survive *a priori* (Franklin and ffrench Constant, 2017).

Nonetheless, there are clear benefits that come with remyelination, making it an important biological process to basic neuronal function and survival.

1.2.3 The stages of remyelination

By virtue of the toxin-demyelination models developed, the various stages leading to remyelination have been characterised in detail. As with any regenerative process, remyelination comprises four distinct yet overlapping stages (Figure 1.2). Firstly, there is an initial inflammatory response to the injury, immediately followed by the recruitment of resident progenitor cells. Consequently, the progenitors differentiate into post-mitotic residents of the tissue of interest and finally they execute the endogenous repair required (Levine and Reynolds, 1999; Fancy et al., 2004). The correct outcome of each of these phases is fundamental if remyelination is to be carried out to completion and for its functional benefits to be achieved.

Inflammation

Primary demyelination leads to a quick inflammatory response driven by resident microglia and macrophages, and depletion of inflammatory cells within the lysolecithin lesion at the

early stages of remyelination resulted in remyelination of fewer axons three weeks after lesion induction (Kotter et al., 2001). There was no effect on the repair outcome if the depletion was delayed to the second phase of remyelination, suggesting that the innate immune response is essential at the initial stages of the process (Kotter et al., 2001). The importance of inflammation in achieving successful remyelination was further shown by gain-of-function experiments where oligodendrocyte progenitor cells (OPC) were transplanted in the retina of adult rats. Inflammation induction in retinal areas via the administration of zymosan, a TLR-2 ligand, resulted in increased differentiation of transplanted OPCs and myelination of retinal ganglion cell axons compared to OPCs transplanted in non-inflamed areas (Setzu et al., 2006). This model provides further evidence in support of the view that an inflammatory response associated with demyelination is a salient trigger in activating OPCs and creating conditions favourable to remyelination. Efficient remyelination by the innate immune system involves two fundamental functions: myelin debris clearance and the secretion of factors important for the lesion environment and for OPC recruitment (Messersmith et al., 2000; Murtie et al., 2005; Kotter et al., 2006; Zhou et al., 2006). It has been shown that myelin debris prevents OPC differentiation *in vitro* (Robinson and Miller, 1999), observations which have also been confirmed *in vivo*. Administration of myelin debris to EB lesions in rats negatively impacted remyelination efficiency without affecting neither macrophage nor OPC recruitment (Kotter et al., 2006). Therefore, clearance of myelin debris via phagocytosis is a crucial step for oligodendrocyte formation and consequently remyelination. *In vitro* observations that treatment of primary OPCs with microglia-conditioned media could modulate their behaviour also suggest a phagocytic-independent role for the innate immune system in remyelination (Miron et al., 2013). Miron et al. (2013) found the growth factor activin-A to be an important modulator of OPC differentiation in the conditioned media experiments. Similarly, other factors produced by the innate immune system during this inflammatory stage have been discovered to affect remyelination. These include transforming growth factor (TGF) β, insulin-like growth factor and endothelin 2 (McMorris and Dubois-Dalcq, 1988; McKinnon et al., 1993; Yuen et al., 2013), which are further summarised by McMurran et al. (2016). Contrary to the belief that CNS inflammation always has a negative impact, the above evidence indicates that, in the context of remyelination, a robust inflammatory response is necessary in order for regeneration to occur successfully.

OPC activation and recruitment

Following the onset of inflammation OPCs are recruited into the lesion. In order for OPCs to be mobilised, they first need to become *activated*; this term is used to describe the state of OPCs upon disruption of their homeostatic environment (Levine and Reynolds, 1999; Fancy

et al., 2004; Nakatani et al., 2013; Moyon et al., 2015). What exactly determines OPC activation is still unknown; however, it has been hypothesised that tissue damage is initially sensed by microglia and astrocytes, which in response release an array of factors into the tissue microenvironment. These are in turn sensed by resident OPCs and lead to a shift in the signalling pathways activated within the progenitors, changing their transcriptional profile (Nakatani et al., 2013; Moyon et al., 2015). Microarray analysis of neonatal, adult OPCs and oligodendrocytes from homeostatic white matter, as well as OPCs in demyelinated lesions, revealed that homeostatic OPCs resemble oligodendrocytes in their transcriptional profile more closely than neonatal OPCs. Instead, activated OPCs in demyelinated lesions transcriptionally resemble their neonatal antecedents (Moyon et al., 2015). Therefore, in an injury setting, OPCs shift from a homeostatic to an activated state which closely resembles neonatal OPCs (Fancy et al., 2004; Moyon et al., 2015). The purpose of this activation is to make OPCs more responsive to migratory and proliferative signals involved in recruiting them to the lesion. Numerous factors have been shown to contribute to OPC recruitment, many of which have been shown to be released by the innate immune system, reactive astrocytes as well as cells of the vasculature (Messersmith et al., 2000; Spassky et al., 2002; Arai and Lo, 2009; Lindner et al., 2015; Moyon et al., 2015; Tsai et al., 2016). Recruitment is successful only if there is a fine interplay between migratory/proliferative signals and cues preventing OPC differentiation. The proliferative response is controlled by numerous mitogens including Platelet-derived growth factor (PDGF) and FGF2. Increasing levels of PDGF-A result in higher numbers of OPCs in the lesion whilst knockouts of their receptors lead to a decrease in the density of progenitors (Woodruff et al., 2004; Murtie et al., 2005). Semaphorins, which are important guiding cues for axons to find to their synaptic target in neurodevelopment, have also been shown to influence OPC migration (Piaton et al., 2011; Boyd et al., 2013). Gain and loss of function experiments both *in vitro* and *in vivo* have shown that semaphorins 3A and 3F, for which OPCs express the receptors, influence the recruitment of adult OPCs to demyelinated lesions (Williams et al., 2007; Piaton et al., 2011; Boyd et al., 2013). Among these cues we also find signals inhibiting OPC differentiation such as Notch, canonical Wnt signalling and the regulation of cyclin dependent kinases (CDK): these prevent premature cell cycle exit, ensuring that the correct number of OPCs in the lesion is reached in order for remyelination to occur (Fancy et al., 2009; Zhang et al., 2009; Caillava et al., 2011).

OPC differentiation and remyelination

Once OPCs are recruited into the demyelinated lesion, a transition to the differentiation stage occurs. Research has concentrated in teasing out the signals involved in promoting OPC

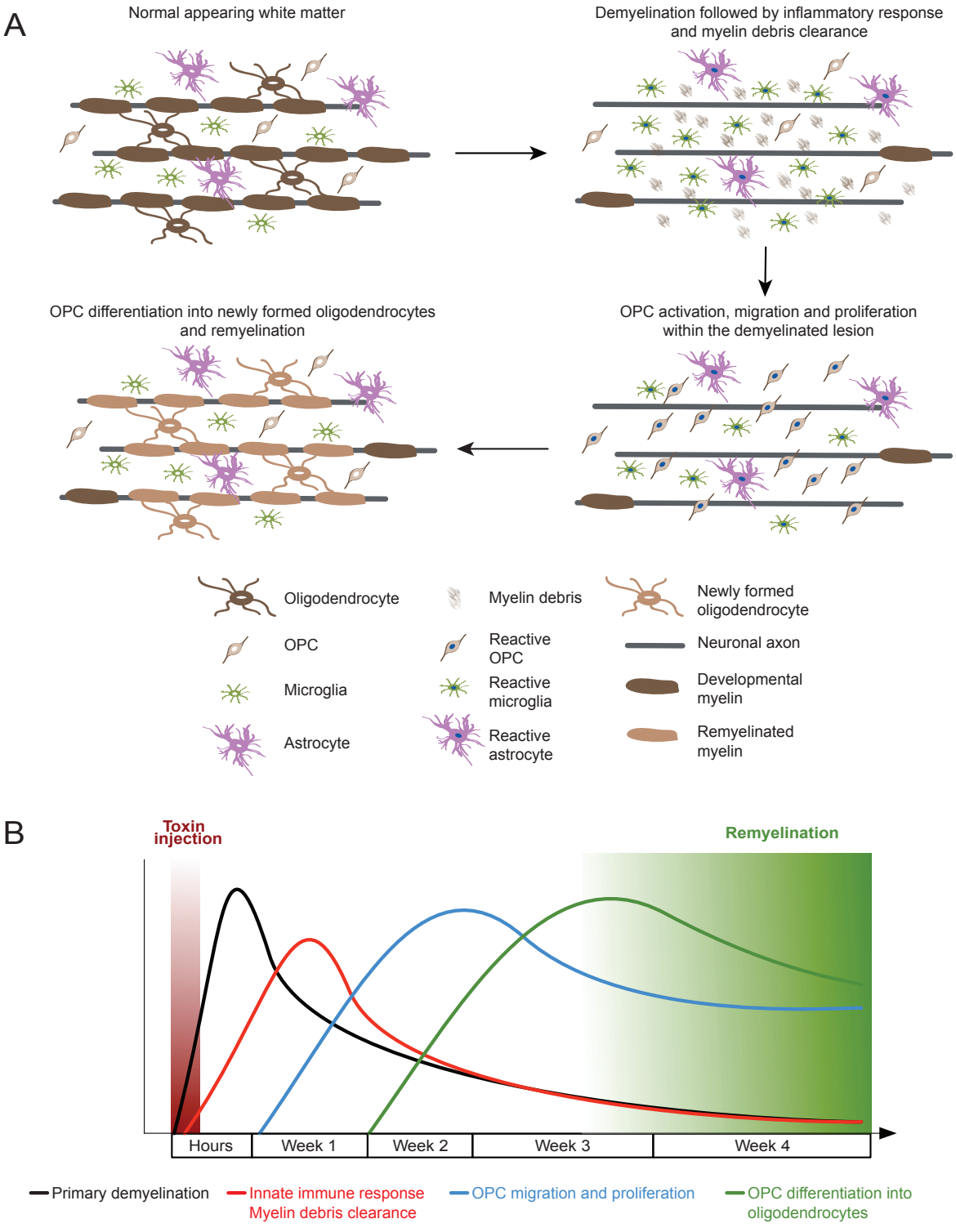


Fig. 1.2 Schematic showing the stages of remyelination. (A) Upon primary demyelination macrophages and microglia mount an inflammatory response, clearing myelin debris via phagocytosis. Reactive microglia and astrocytes activate neighbouring OPCs which migrate and proliferate in the lesion. Upon reaching a certain density, OPCs start to differentiate creating newly formed oligodendrocytes that will restore the lost myelin. (B) A timeline of the events following toxin injection in a toxin-induced demyelination model. Adapted from (Franklin and ffrench Constant, 2008).

differentiation both at the molecular and cellular levels. At the molecular level, terminal differentiation of progenitors usually involves a two-step mechanism, where they firstly exit the cell cycle (usually via antagonism of CDKs) and subsequently increase the expression of cell-specific genes involved in differentiation (Ruijtenberg and van den Heuvel, 2016). As with any other progenitor cell, it has been shown that OPCs lacking the CDK inhibitor p27 continue to proliferate at the expense of differentiation *in vitro* (Casaccia-Bonnefil et al., 1997). Other common stem cell-specific factors are involved in controlling OPC proliferation, including important transcriptional regulators such as E2F1 and *c-Myc* (Magri et al., 2014b,a). E2F1 targets the expression of cell cycle genes and its inhibition is essential to promote cell cycle exit. In accordance with this, mouse gliomas, tumours resulting from OPCs, present an increased expression of E2F1 (Magri et al., 2014b) The expression of the proto-oncogene *c-Myc* has been used to control reprogramming of cells (Thier et al., 2012), suggesting its key role in determining cell fate. *c-Myc* also controls important cell cycle genes, and its silencing results in their promoter regions to be silenced via chromatin methylation and compaction, whilst promoting genes involved in differentiation (Magri et al., 2014a). More recent interest has developed in the involvement of specific transcription factors determining chromatin modification and transcription of oligodendrocyte differentiation-specific genes, as shown by Moyon et al. (2016).

At the cellular level, an example of a determining factor shown to promote OPC differentiation is the cell density within a given space. *In vitro* assessment of the impact of cell density on differentiation in development revealed that OPC differentiation is induced once a specific density is reached (500-600 cells/mm^2). This is the case regardless of the initial seeding density of the cells (Rosenberg et al., 2008). Density could impact cell fate in a number possible ways: a high number of OPCs could prevent proliferative mitogens from reaching a portion of the cells, which will then become oligodendrocytes, they may inhibit further proliferation via contact-dependent inhibition as in other cell types, or the spatial constraints may mechanically induce OPC differentiation (Rosenberg et al., 2008; Hughes et al., 2013; Watt and Huck, 2013). This model could apply in an adult *in vivo* setting, whereby a lesion requires a certain density of OPCs to ensure complete remyelination, making OPC density crucial for differentiation.

The picture we are presented with is a complex array of signals apparently disconnected from each other, and future research has the aim of piecing this information together for a complete picture of what is happening *in vivo*. The result of OPC differentiation is the production of new oligodendrocytes which will extend their processes, make contact with demyelinated axons and subsequently wrap them with newly formed myelin sheaths (Zawadzka et al., 2010).

1.3 Oligodendrocyte lineage cells throughout life

1.3.1 The role of OPCs in development and adulthood

OPCs arise during embryonic development from three spatiotemporal distinct waves originating from the neuroepithelium (Kessaris et al., 2006). In the murine brain, the first wave of OPCs is derived from the ventral telencephalon, specifically the medial ganglionic eminence and the anterior entopeduncular, at embryonic day (E) 11.5. The second wave is also derived ventrally, from the lateral and caudal ganglionic eminence at E16.5. The first two waves spread to populate the whole telencephalon until the final dorsally derived OPC wave arises postnatally. Subsequently, dorsal OPCs populate the dorsal areas of the telencephalon, eliminating OPCs derived from the first ventral wave (Kessaris et al., 2006). In the adult brain, dorsally derived OPCs mainly populate the cortex and corpus callosum, whilst other brain areas are mainly populated by ventrally derived OPCs from the second wave (Kessaris et al., 2006). OPCs clearly present developmental heterogeneity. However, whether this translates to a functional heterogeneity is still to be determined.

Myelination occurs mainly postnatally in the first 10 weeks in rodents, and the first 10 years in humans (Yeung et al., 2014). OPC numbers drop in the first years of life and stabilise around five years of age, whilst production of new oligodendrocytes follows the inverse pattern, plateauing closer to 10 years of age (Yeung et al., 2014). This is a critical age for human development, as exponential learning of both motor and cognitive skills occurs during this period. In fact, the onset of myelination correlates with the development of numerous abilities, including motor coordination, fine motor tuning and cognitive skills. The impact that myelination can have on development is reinforced by the observation that myelin deficits arise in many common developmental disorders such as autism and attention deficit hyperactivity disorder (Bercury and Macklin, 2015), as well as in psychiatric conditions like Schizophrenia (Uranova et al., 2004; Windrem et al., 2017). Even though the bulk of myelination occurs in the first years of life, increase in white matter volume persists throughout adulthood (Lebel et al., 2012; Hill et al., 2018; Hughes et al., 2018). This raises an important question: do OPCs have a function in CNS homeostasis and what could this entail? A reasonable assumption would be the replacement of old oligodendrocytes with new ones as for post-mitotic cells in other tissues. However, there is no current evidence of oligodendrocytes undergoing apoptosis during homeostasis, and post mortem experiments looking at the integration of radioactive carbon in individuals of different ages show that there is some but little oligodendrocyte turnover throughout life (Yeung et al., 2014). This has also been observed in the mouse, where *in vivo* two-photon imaging used to monitor oligodendrocyte formation in *MOBP-EGFP* transgenic mice revealed minimal turnover of

oligodendrocytes in the adult mouse cortex (Hughes et al., 2018). Nevertheless, new myelin is continually produced in both the adult human and rodent, suggesting that already existing oligodendrocytes may be responsible for the increase in myelin volume with age (Yeung et al., 2014; Hill et al., 2018). Interestingly, learning new skills, such as juggling or playing a musical instrument for humans, as well as sensory enrichment for mice, have been shown to alter white matter volume (Bengtsson et al., 2005; Scholz et al., 2009; Hughes et al., 2018). Despite the white matter being full of oligodendrocytes, an increase in its volume may not necessarily be due to an increase in newly formed oligodendrocytes, but rather be due to production of more myelin by pre-existing oligodendrocytes or to an increase in neurogenesis which consequently requires myelin for new neurons. McKenzie et al. (2014) have shown the implication of adult *de novo* myelination in mice that learnt motor skills such as running on a complex wheel. Upon learning this skill, an increase in the number of new oligodendrocytes produced was observed. Inhibition of oligodendrocyte formation and adult myelination by deleting myelin regulatory factor (*Myrf*), mice found greater difficulty in learning new motor skills (McKenzie et al., 2014). Subsequently, a rapid production of newly formed oligodendrocytes was observed in the early hours following the introduction of the mice to the wheel, coinciding with synaptic changes required for learning (Xiao et al., 2016). This suggests that OPCs and adult oligodendrogenesis contribute to the process of learning and, even though this research is in its early days, it appears that they play a much more active role than previously thought.

Further research is required to fully understand the possible roles carried out by OPCs in homeostasis, and as of today the most well-defined role for adult OPCs remains their involvement in remyelination. Due to this, ever since their first discovery by Raff et al. (1983), OPCs have been at the centre of CNS regenerative research. This is because they are the largest CNS progenitor population, making up 5%-8% of the total adult CNS, and are spread evenly throughout the grey and white matter (Pringle et al., 1992), but whether they are to be considered legitimate adult stem cells is still subject of debate in the field.

1.3.2 Are OPCs *bona fide* stem cells?

For any adult stem cell to be considered as such, it needs to present certain properties and capabilities. These include self-renewal, mitotic quiescence, multipotency, enabling tissue regeneration as well as having a niche (Crawford et al., 2014).

OPCs are able to proliferate throughout the entire lifespan of an organism (Young et al., 2013). Numbers of OPCs *in vivo* are similar between animals of different age, suggesting that a stable OPC density is maintained throughout lifetime (Rivers et al., 2008). Ablation of single OPCs under homeostatic conditions causes neighbouring progenitors to proliferate

and replace the missing cell, ensuring maintenance of the correct cell density (Hughes et al., 2013). Furthermore, multiple episodes of demyelination do not result in a depletion of progenitors, meaning that the progenitor pool continually self-renews and is never exacerbated (Penderis et al., 2003).

OPCs have been reported to exhibit asymmetrical division, whereby a stem cell divides to generate a daughter cell that will retain stem cell identity, thereby achieving self-renewal, and a daughter cell destined to differentiate. This is obtained by the asymmetrical division of cell fate determinants at cytokinesis, where the daughter cells receive unequal fate determinants thereby conditioning their distinct cell fates (Morrison and Kimble, 2006). True asymmetrical division occurs at the basal lamina of tissues, in an apical-basal orientation, as in the case of skeletal muscle satellite cells. Upon satellite cell division, the daughter cell closer to the basal surface retains stem cell identity, whilst the more apical daughter cell presents transcription factors involved in myogenic differentiation (Kuang et al., 2007). Although OPCs present different cell fates as discussed further below, this does not indicate true asymmetrical division. Indeed, OPCs have been reported to asymmetrically divide cell fate determinants such as the proteoglycan NG2. Monitoring of the segregation of NG2 upon OPC division showed that out of the two daughter cells, the one retaining NG2 would continue to self-renew, whilst the daughter lacking NG2 would differentiate (Sugiarto et al., 2011). However, further evidence is required for OPCs undergoing true asymmetrical division, as *in vivo* time-lapse studies demonstrate that the vast majority of OPCs differentiate without any prior cell division event (Hughes et al., 2013). Additionally, the absence of a true basal lamina in their microenvironment suggests that OPCs may divide in a planar orientation raising an important question on how OPCs achieve polarity upon cell division (Crawford et al., 2014).

OPCs are able to regenerate whole myelin sheaths after demyelination, and there is substantial evidence that new oligodendrocytes come from OPCs rather than from already existing oligodendrocytes (Zawadzka et al., 2010; Crawford et al., 2016). However, OPCs are not only restricted to the oligodendrocyte lineage. The first *in vitro* studies immediately pointed towards the bipotent nature of OPCs. By isolating these cells from the rat optic nerve, Raff et al. (1984) observed that neonatal OPCs had the potential to differentiate either into oligodendrocytes or type II astrocytes, depending on the media they were exposed to. Even though OPCs may present the potential to differentiate into astrocytes, there is very little evidence of this phenomenon *in vivo*, where reactive astrocytes in lesions derive from pre-existing astrocytes (Zawadzka et al., 2010). On the other hand, fate-mapping analysis in the context of demyelination found OPCs to give rise to Schwann cells capable of remyelinating CNS axons (Zawadzka et al., 2010). CNS Schwann cells arise only in lesion areas which lack astro-

cytes; the inhibitory effect of astrocytes on Schwann cells had already been observed *in vitro*, and the use of a signal transducer and activator of transcription 3 knockout mouse model, in which astrocytic activation was abrogated, resulted in a decreased oligodendrocyte-mediated remyelination and an increase in Schwann cell remyelination upon toxin-induced demyelination (Guenard et al., 1994; Black et al., 2006; Zawadzka et al., 2010; Monteiro de Castro et al., 2015).

OPCs have been described to be evenly spread throughout the entire CNS of organisms (Dawson, 2003). These observations come from immunohistochemistry studies of various brain regions, where OPCs were immunolabeled with antibodies targeting the OPC marker NG2 (Dawson, 2003). The even distribution of OPCs throughout the CNS suggests that they may lack a stem cell niche unlike other stem cell types.

Adult NSCs, for example, reside in two niches: the subventricular zone and the subgranular layer. Signals from the neurogenic niche influence the proliferation of NSCs, or their differentiation into mature neurons when required (Song et al., 2002; Shen et al., 2004; Alvarez-Buylla et al., 2008).

NSCs in these niches are involved in tissue homeostasis for the olfactory bulb and memory formation, and their role could be the reason as to why they are only restricted in these specific areas (Winocur et al., 2006; Curtis et al., 2007). OPCs, on the other hand, are needed for remyelination, a process which may be required in any area of the CNS. However, all previous studies have assessed staining in a restricted plane limited to brain slices, potentially compromising the observations and conclusions that can be made. Recent unpublished efforts by Neumann and Segel in the Franklin laboratory aim to disentangle whether there is indeed one or multiple OPC niches throughout the murine CNS.

OPCs, therefore, do have stem cell capabilities such as multipotency, self renewal and the ability to generate a progeny of oligodendrocytes which carry out remyelination, even after multiple rounds of demyelination (Penderis et al., 2003).

1.3.3 Oligodendrogenesis involves transcriptional and morphological changes in OPCs

Despite their multipotency, oligodendrocytes remain the main progeny of OPCs (Zawadzka et al., 2010). The linear transition from OPC to oligodendrocyte involves a series of changes at the morphological, chromatin remodelling and transcriptional levels, all of which are essential for the creation of a completely new cell with a different physiological role. OPC differentiation *in vitro* involves dramatic morphological changes, starting with a bipolar OPC which, over time, will become a multi-processed cell. Eventually, the cell will grow and

spread sheaths on the surface it is cultured on. If the oligodendrocytes *in vitro* are presented with something to myelinate, such as axons (Gardner et al., 2012), nanofibres (Lee et al., 2013; Bechler et al., 2015) or micropillars (Mei et al., 2014), they will wrap their sheaths around them, thereby becoming a myelinating oligodendrocyte. These morphological changes are accompanied by the expression of stage-specific markers (Pringle et al., 1992; Schumacher et al., 2012; Xiao et al., 2016), allowing for a more precise distinction between the various stages.

Markers for the entire oligodendrocyte lineage, such as Olig2 and Sox10, are essential to the differentiation program (Yu et al., 2013; Hornig et al., 2013). Both are transcription factors shown to control the transcription of genes essential to OPC differentiation. Olig2 has been shown to be fundamental in both specification of the oligodendrocyte lineage as well as its differentiation (Takebayashi et al., 2002; Yue et al., 2006). ChIP-sequencing analysis has revealed that Olig2 recruits a SWI/SNF chromatin remodeller to enhancer regions controlling the expression of OPC differentiation regulators such as *Myrf*, *Olig1* and *2*, *Sox10* and *Zfp191*. The recruitment of SWI/SNF makes the chromatin in these areas accessible, thereby allowing their transcription and promoting differentiation (Yu et al., 2013). Sox10 is also necessary for terminal differentiation of OPCs, as its loss prevents differentiation and CNS myelination. Like Olig2, Sox10 also binds to enhancer regions of OPC differentiation genes such as *Myrf*, thereby controlling their expression (Hornig et al., 2013). Olig2 and Sox10 are therefore essential to driving the expression of transcription factors that in turn will allow for the production of myelin proteins and OPC differentiation.

As well as transcription factors, chromatin modification itself is crucial for OPC differentiation to occur, as it controls whether a gene is accessible for transcription or not. Numerous histone deacetylases (HDAC) have been reported to influence OPC differentiation or specification, including HDAC 1, 2, 3 and 11 (Ye et al., 2009; Zhang et al., 2016a; Liu et al., 2009). Furthermore, components of chromatin remodelling complexes such as BRG1-dependent SWI/SNF, SIRT1, and p300 have all been reported to influence OPC differentiation, either by inhibiting or promoting it (Yu et al., 2013; Jablonska et al., 2016; Zhang et al., 2016a)

The inhibition of negative regulators of differentiation is also a crucial aspect of oligodendrogenesis. OPCs have high levels of transcription factors involved in maintaining stem cell identity, such as Hes5, Id2, Sox5 and 6, which prevent differentiation and myelination (Samanta and Kessler, 2004; Liu et al., 2006; Stolt et al., 2006). Id2 is downstream of the oligodendrogenesis inhibitor bone morphogenic protein (BMP) 4, and it has been suggested that Id2 prevents Olig1/2 from exerting their function by directly interacting with them (Samanta and Kessler, 2004). Hes5, on the other hand, is downstream of the Notch signalling pathway. Although the role of Notch in OPCs remains controversial, Notch lig-

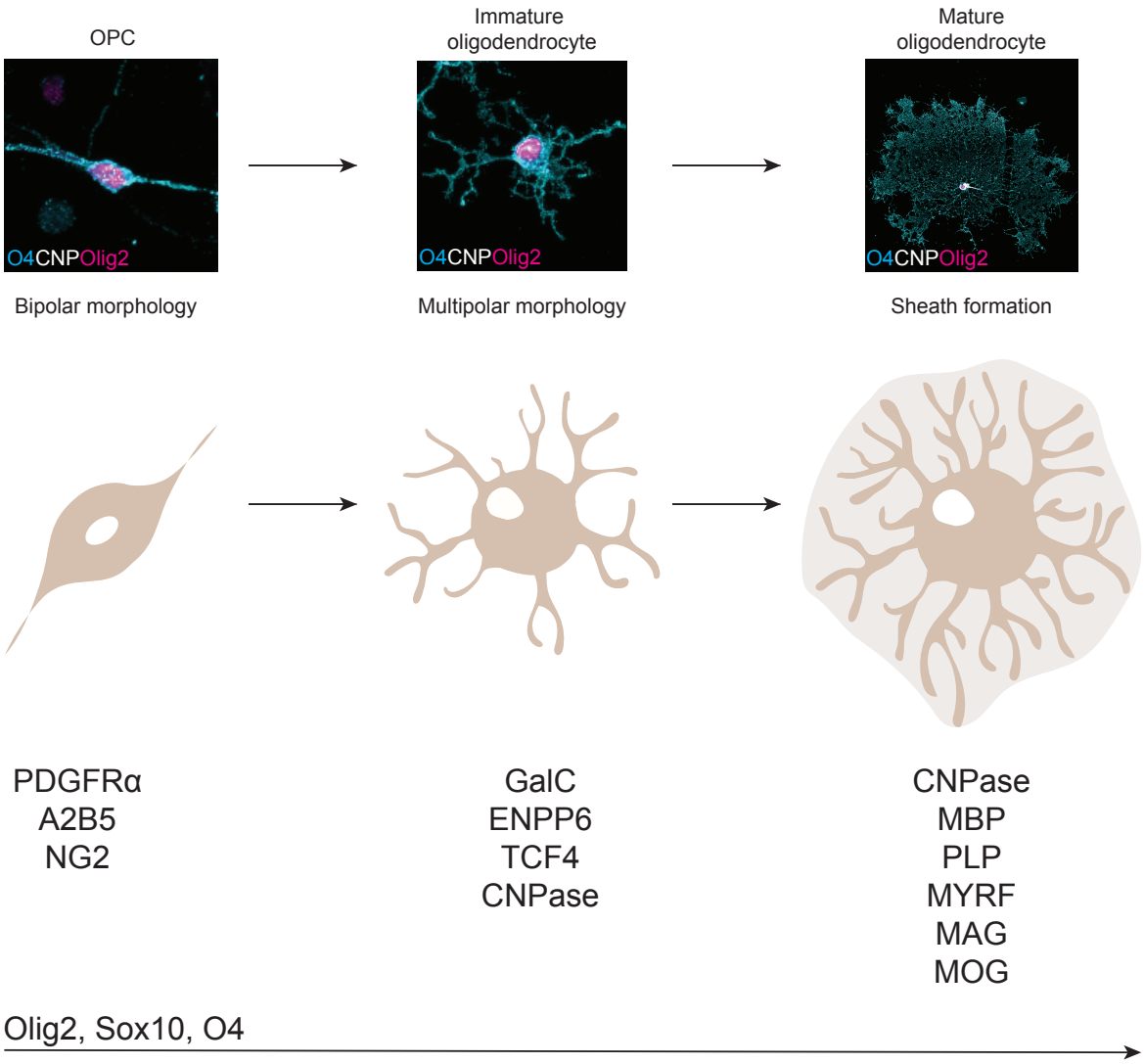


Fig. 1.3 The oligodendrocyte lineage differentiation timeline. The top panel shows immunocytochemistry staining of an OPC, immature and mature oligodendrocyte *in vitro*. The cells have been stained with the lineage markers Olig2 and O4 to show their morphology. OPCs have a simple bipolar morphology, and as they differentiate they become multiprocessed and grow in size, until they become mature and lay down sheaths in culture. Differentiation entails changes in the gene expression profile of the cell as it progresses through the lineage. The most commonly used stage-specific markers are shown, as well as markers used to identify the entire lineage. The process of differentiation consists in the same cell transitioning between one phase to the next, thereby the expression of stage-specific markers is not a binary event, but rather a smooth transition. Usually a combination of the above markers is used to assess different stages of the lineage. The immunocytochemistry pictures above are not the same magnification.

ands have been shown to inhibit OPC differentiation (Wang et al., 1998), and this may be due to their induction in *Hes5* expression which in turn will prevent Sox10 from exerting its function (Liu et al., 2006). As well as Notch signalling, canonical Wnt signalling is involved in preventing OPCs from differentiating, where the transcriptional complex formed by β-catenin and Tcf7l2 prevents OPC differentiation (Fancy et al., 2009).

Therefore, despite being a seemingly linear process, oligodendrogenesis is highly demanding, both at the transcriptional and metabolic level. Negative regulators of differentiation need to be inhibited while positive regulators need to be promoted, and these include, but are not limited to, the ones highlighted above. The pathways governing these events are tightly controlled and intertwined, giving rise to a highly complex molecular network governing oligodendrogenesis.

1.4 Ageing leads to remyelination failure

1.4.1 The hallmarks of ageing

Ageing is the time-dependent loss of homeostatic mechanisms resulting in the functional decline of an organism. Impaired homeostasis implies tissue dysfunction, which consequently makes us more susceptible to a whole array of diseases with increasing age. This is an interesting observation as it implies that ageing is the primary risk factor for numerous diseases, including neurodegeneration, heart conditions, diabetes, cancer and many more (Jaul and Barron, 2017). Consequently, the research in this field aims at finding ways to slow the rate of ageing, maintaining cells and tissues in a juvenile-like state, in order to achieve increased health and an extended lifespan.

Why and how we age are amongst the biggest mysteries in modern biology, and even though the conceptualisation of an ageing organism can be difficult, the identification of several molecular and cellular hallmarks have helped in understanding its essence (López-Otín et al., 2013). These hallmarks can be broadly divided into two categories: intracellular and extracellular hallmarks. Intracellularly, ageing cells present an accumulation of damage in both nuclear and mitochondrial DNA, telomere attrition, numerous epigenetic alterations, mitochondrial dysfunction, cellular senescence and deregulation of proteostasis and nutrient sensing (Koga et al., 2011; Faggioli et al., 2012; Forsberg et al., 2012; Tsurumi and Li, 2012; Park and Larsson, 2011; Johnson et al., 2013). Extracellular hallmarks present altered intercellular communication as well as changes in the ageing microenvironment that can negatively impact resident progenitors (López-Otín et al., 2013). OPCs are no exception

to this, and their functional deterioration is caused by intrinsic and extrinsic factors in the ageing microenvironment, both of which culminate in the OPC ageing phenotype.

1.4.2 Remyelination efficiency declines with age

Despite remyelination going to completion, its efficiency declines with increasing age (Gilson and Blakemore, 1993; Shields et al., 1999; Sim et al., 2002). Ageing, in fact, gives rise to changes affecting the intrinsic properties of OPCs as well as factors in their extrinsic microenvironment, culminating in an inability of the progenitors to carry out their regenerative role. I have previously described the events involved in remyelination, and a delay in the process could be the result of either impaired OPC recruitment and/or an inability of the recruited OPCs to differentiate within a lesion. So where in these processes is the bottleneck causing unsuccessful remyelination in ageing? There is evidence for defects in both the recruitment and differentiation of OPCs in ageing animals (Sim et al., 2002).

OPC repopulation in toxin-induced lesions is delayed in old animals compared to their young counterparts (Sim et al., 2002). Demyelinated lesions in aged rats accumulate progenitors mainly at their border rather than being evenly spread throughout, and are unable to match the OPC density displayed by younger animals at early recruitment time points (Sim et al., 2002). An explanation could be that OPC numbers decline with increasing age, making the available number of progenitors to recruit lower than in young organisms. Despite the ability to self-renew, an age-related decline in stem cell numbers has been observed also in the case of NSCs, for example, (Maslov et al., 2004), making this a fair assumption. However, evidence so far points towards OPC density remaining constant throughout life, at least in the brain areas assessed to date, and overall, a high number of OPCs reside in the aged rodent brain (Sim et al., 2002; Doucette et al., 2010; Rivers et al., 2008). Nevertheless, if the number of OPCs does not change with ageing, other factors must be impairing their recruitment. Studies on OPC cell cycle dynamics revealed that their cell cycle slows down with age progression, presumably due to a lengthened G1 phase (Psachoulia et al., 2009; Ruckh et al., 2012; Young et al., 2013). Besides the change in OPC cell cycle dynamics, the specific implications of which have not yet been directly assessed in remyelination, it cannot be excluded that signals shown to influence OPC proliferation and recruitment may also be altered with age. The latter is a feasible possibility, as the expression of growth factors such as PDGF-A, IGF-I, and TGF-β1 decreases with age, indicating a change in how the ageing innate immune system responds to demyelination (Hinks and Franklin, 2000). It is therefore possible to hypothesise that the slow recruitment and proliferation of OPCs in old animals may be a consequence of altered cell cycle dynamics of aged progenitors and impaired intercellular communication in the aged microenvironment.

If lack of OPC numbers in the lesion was the main cause of impaired remyelination in ageing, then increasing OPC recruitment should restore successful remyelination. Overexpressing PDGF-A in experimentally demyelinated lesions enhanced the OPC numbers recruited. However, this did not have any effect on neither the timing or the extent of remyelination (Woodruff et al., 2004). Sim et al. (2002) further observed that, as well as delayed migration, aged lesions presented a delay in OPC differentiation. As impaired epigenetic regulation of gene expression is a key hallmark of ageing cells, and HDACs exert a salient role in OPC differentiation, their ability to regulate this process in old animals was assessed. Overall, cuprizone-induced demyelination in young animals is followed by a rapid increase in HDAC1 and HDAC8 expression in OPCs, a process essential to the control of Sox2 expression, which in turn inhibits differentiation. Instead, aged OPCs present decreased expression of HDAC1 and 8 and increased expression of Sox2 following demyelination (Shen et al., 2008). This study serves as an example of the impaired and inefficient epigenetic regulation exhibited by aged progenitors, resulting in the persistent expression of genes preventing OPC differentiation. The study of intrinsic factors governing OPC differentiation can be complicated due to the difficulty of isolating and culturing aged progenitors *in vitro*, and only recently are we beginning to explore the multitude of intrinsic factors governing the transcriptional program of OPC differentiation. On the other hand, the extrinsic factors impairing progenitor differentiation in aged animals have been better characterised, the majority of which seem to be mediated by an aged immune system. Aged animals present a delayed immune response following demyelination. The importance of an immediate immune response to demyelination has been previously outlined, and its delay implies a shift in the timeframe of the entire remyelination process. It may therefore be that, with ageing, remyelination is not impaired *per se*, but may rather be extensively delayed. However, aged macrophages also present impaired phagocytosis capability, resulting in inefficient myelin debris clearance (Natrajan et al., 2015; Lampron et al., 2015). Myelin debris has been shown to be a key inhibitor of OPC differentiation both *in vitro* and *in vivo* (Kotter et al., 2006), and its persistence in aged lesions due to lack of clearance could be the reason for impaired OPC differentiation. Further evidence of the importance of a young and efficient immune system in promoting remyelination came from experiments involving heterochronic parabiosis (Ruckh et al., 2012). In these experiments, young mice were joined with aged mice so that they would share their circulation. Toxin demyelination was then induced in the aged animal and remyelination was assessed. The experiment showed that upon exposure to a youthful systemic environment, remyelination was indeed enhanced, with aged OPCs being both more proliferative and presenting higher levels of differentiation compared to the isochronic-old controls. Remyelination was indeed carried out by aged OPCs as there was

no engraftment of young OPCs in the lesions, but a high number of young macrophages were found in the lesion, which correlated with increased myelin debris clearance (Ruckh et al., 2012).

Based on the above, it is clear that a combination of both intrinsic and extrinsic factors contribute to the ageing phenotype of OPCs which, however, remain responsive to exogenous signals promoting their proliferation and differentiation. The parabiosis experiments by Ruckh et al. (2012), as well as the currently unpublished work by Neumann et al. in the Franklin laboratory on the effect of calorie restriction on aged OPCs provide evidence that *in vivo* rejuvenation of OPCs is possible, as extrinsic signals can override the intrinsic progenitor ageing phenotype. This area of research will be fundamental in the development of regenerative therapies as ageing progenitors are clearly different to their younger counterparts.

1.4.3 Multiple Sclerosis is also a disease of age

The majority of neurodegenerative diseases arise in the aged population, establishing a strong causal relationship between ageing and neurodegeneration (Wyss-Coray, 2016). Instead, in the majority of cases MS has an onset in young adulthood, which has set it apart from other neurodegenerative diseases with regards to its age onset. Despite the earlier appearance of the disease, age is a salient factor in influencing both the form of MS affecting the patient and its clinical course (Confavreux and Vukusic, 2006; Sanai et al., 2016).

In 80% of patients, MS presents as relapsing remitting MS (RRMS), which is characterised by episodes of acute inflammation causing neurological impairment, followed by recovery, probably due to remyelination (Compston and Coles, 2008; Franklin and ffrench Constant, 2008). Over time, approximately 65% of the RRMS patients will enter the secondary progressive phase of MS (SPMS) where recovery no longer occurs and disability progressively accumulates. The remaining 20% of cases present as primary progressive MS (PPMS), where the disorder is progressive immediately from the onset (Compston and Coles, 2008). It has been reported that patients over 65 years of age tend to have a progressive course compared to younger patients (Minden et al., 2004). Older patients, therefore, tend to present with PPMS, and older age onset of RRMS results in an earlier conversion to SPMS (Scalfari et al., 2011). Indeed, patients that developed RRMS at the age of 20 halved the risk of an early conversion to SPMS compared to patients that developed RRMS at the age of 40 (Scalfari et al., 2011). Confavreux and Vukusic (2006) showed that MS patients reached specific levels of disability at approximately the same age regardless of disease onset and whether it started as RRMS or PPMS, further suggesting an age-dependent increase in disability accumulation. After reaching the critical switch when disability starts to accumulate, there is no

difference between the PPMS and SPMS patients in reaching the next disability score despite the difference in inflammatory episodes (Confavreux and Vukusic, 2006). This suggests that at this critical point of the disease the accumulation in disability does not tightly depend on the inflammatory state of MS, but likely results from other mechanisms such as the age-dependent inability to remyelinate and to restore function (Franklin and ffrench Constant, 2017).

Although it is hard to assess the course of remyelination in MS patients, pathological data points towards a strong effect of age on the successful outcome of remyelination. As in aged animals, a fraction of MS lesions present little to no OPCs within them (Boyd et al., 2013). It has been shown that MS lesions can aberrantly express chemorepellent cues such as Sema3A and netrin which negatively influence OPC migration, resulting in impaired OPC recruitment (Boyd et al., 2013; Tepavcevic et al., 2014). Furthermore, analysis of chronic MS lesions shows that the majority (60%-70%) contain sufficient OPCs, suggesting that the remyelination rate-limiting factor may indeed be their differentiation rather than recruitment (Wolswijk, 1998; Lucchinetti et al., 1999; Chang et al., 2000). OPCs recruited into these lesions present an immature morphology compared to their young counterparts at equivalent time points, raising the question of what is impairing their differentiation (Wolswijk, 1998; Chang et al., 2000). As with old animals, the aged immune system has a fundamental role and it has been observed that macrophages of MS patients showed impaired phagocytic activity, which is fundamental to myelin debris clearance and allowing OPC differentiation (Natrajan et al., 2015). Overall, the lack of OPC differentiation appears to be the driving cause of remyelination failure in the majority of MS lesions with ageing, and this reflects in MS patients where remyelination impairment may be a deterministic factor in disease progression.

1.4.4 Developing remyelination therapies

It is clear that remyelination carries out beneficial effects involving neuroprotection and promoting neuronal function, and as a consequence its failure leads to irreversible neurological damage. Despite the above, there are no current remyelination enhancing therapies available in the clinic. However, efforts are being invested in the development of such therapies (Franklin and ffrench Constant, 2017). Two major approaches are being taken in trying to enhance remyelination: those involving exogenous or endogenous therapies. Both strategies are being tested in animal models and the choice of therapy should depend on the pathology that the patient presents with (Franklin and ffrench Constant, 2017).

Cell transplantation therapies

Cell transplantation therapies for remyelination involve the transplantation of exogenous cells with the potential to give rise to myelinating cells. This method is particularly valuable in the cases where demyelination occurs as a result of genetic defects, such as in leukodystrophies, as the transplanted cells should take over the role of the original defective OPCs. In fact, glial cell transplantation has been a success in different animal models including in *shiverer* mice (Windrem et al., 2004, 2008) and in dogs with *PLP* mutations (Archer et al., 1997). Both showed the potential of extensive myelination, and Windrem et al. (2008) further reported increased rodent life span and resolution of the typical *shiverer* symptoms. Furthermore, numerous cell types have been successfully transplanted in toxin-induced models of primary demyelination, including OPCs, Schwann cells and olfactory ensheathing cells, all resulting in successful engagement and myelination of demyelinated axons (Groves et al., 1993; Franklin et al., 1996; Bachelin et al., 2005). However, human CNS stem cell transplantation therapies in the context of MS raise major concerns, the biggest being the logic behind the approach. If the majority of MS lesions contain OPCs that retain the ability to remyelinate but fail, mainly due to the hostile aged environment, then transplanted progenitors will likely face the same issue. As mentioned above and further reviewed by Pluchino et al. (2004), numerous candidates have been successfully transplanted in animal models; however, the source of primary cells remains a matter of ethical discussion. The advent of induced pluripotent stem cells (iPSC) has certainly aided in these discussions, as the patient's somatic cells can be used to generate the desired cell type, also minimising the risk of graft rejection (Xie et al., 2016). Indeed, the engraftment of OPCs derived from human iPSCs into neonatal *shiverer* mice resulted in successful myelination and increased life span (Wang et al., 2013). However, the protocols to obtain these cells are still inefficient and protracted, and the genomic instability of iPSCs leads to the risk of tumour formation (Steinbeck and Studer, 2015; Xie et al., 2016). Transplants are highly invasive and complicated procedures with a further risk of haemorrhage and cell aggregate formation (Steinbeck and Studer, 2015). This operation would probably need to be carried out every time a new lesion arises in the patient, leading to multiple rounds of transplants, further increasing the risk of complications in these patients.

Enhancing endogenous remyelination

An appealing alternative is the development of endogenous remyelination therapies, which aim at enhancing remyelination by the already resident OPCs. It is clear that myelination and remyelination are controlled by a variety of positive and negative regulatory factors, and

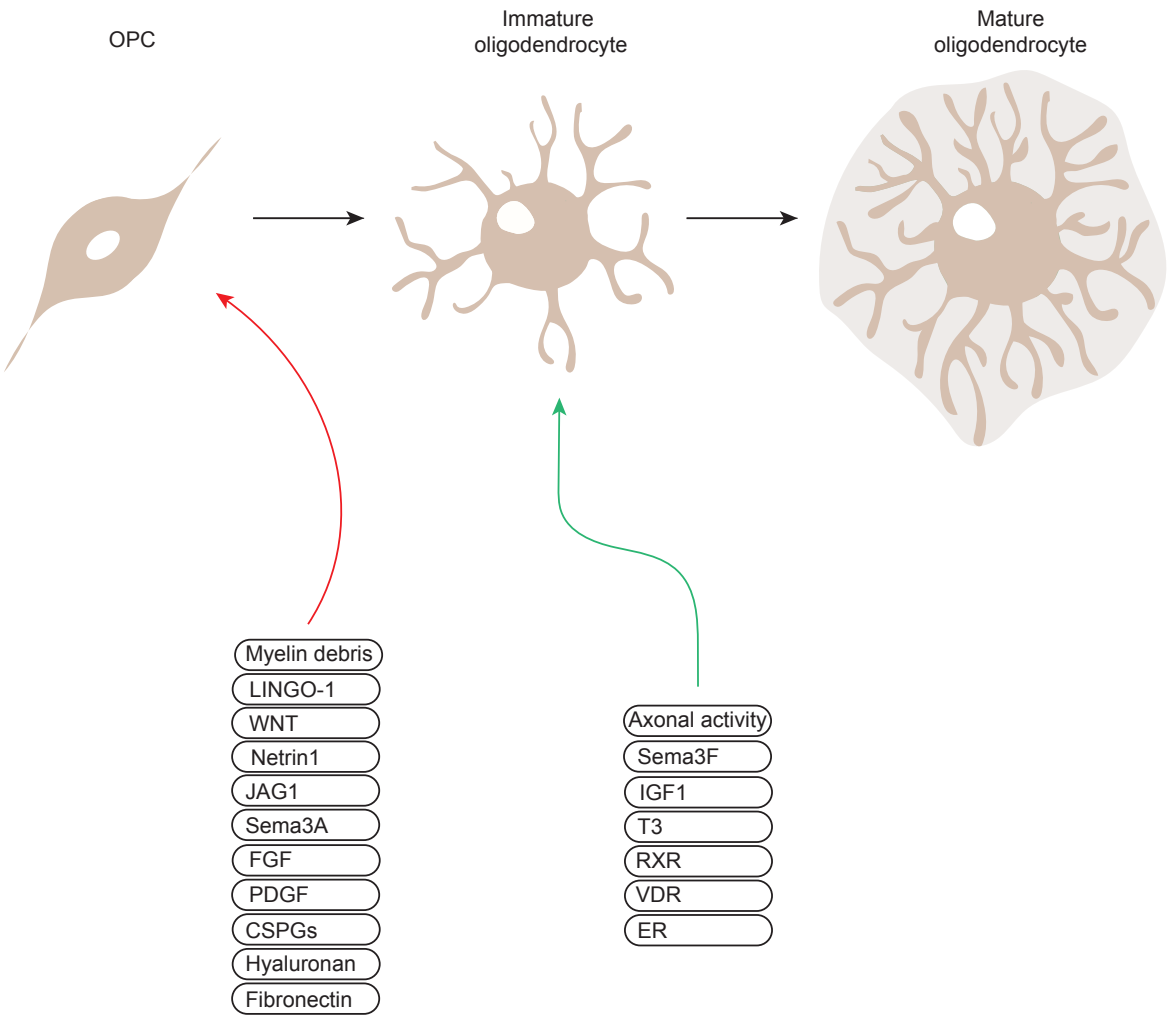


Fig. 1.4 OPC differentiation is a process affected by numerous factors. The factors can be split into two categories: those preventing OPC differentiation and those promoting OPC differentiation. Some of these have been shown to also be involved in remyelination and for this reason they are currently being studied as potential targets for future remyelination therapies.

the aim of such therapies is to understand how these factors can be targeted to manipulate the remyelination outcome. With this aim, remyelination therapies take one of the following two approaches: the direct promotion of OPC differentiation, or the inhibition of negative regulators in the lesion environment (Franklin and ffrench Constant, 2017).

Compound screenings and micropillar assays successfully identified various antagonists of the M1 muscarinic acetylcholine receptors (mAchR) to increase OPC differentiation and micropillar wrapping (Deshmukh et al., 2013; Mei et al., 2014). OPCs express mAchRs, and *M1 mAchR* knockout specific to the oligodendrocyte lineage showed accelerated kinetics of co-culture myelination. Treatment with the antimuscarinic clemastine also accelerated remyelination in toxin models and attenuated EAE symptoms (Mei et al., 2016a). Clemastine has been used for the clinical study ReBUILD (NCT02040298), which was successful in reducing visual evoked potentials and improving low-contrast visual acuity in RRMS patients with episodes of optic neuritis. ReCOVER (NCT02521311) is aimed at patients with acute optic neuritis and is currently recruiting (Plemel et al., 2017). Compound screenings also revealed other medications promoting myelination and remyelination, such as clobetasol and miconazole, involved in activation of Glucocorticoid receptor (GCR) and the ERK1/2 pathway respectively (Najm et al., 2015). K-opioid receptors, Oestrogen receptor (ER), Thyroid Hormone receptor (THR), and glycogen synthase kinase 3 (GSK3) are some of the other targets that have been shown to modulate OPC differentiation *in vitro* and *in vivo* (Mei et al., 2016b; Gonzalez et al., 2016; Zhang et al., 2016b; Medina-Rodríguez et al., 2017; Plemel et al., 2017). Despite the initial success, a potential flaw of these studies is the use of cell lines, iPSCs or young primary cells for their *in vitro* work and young animals for their *in vivo* assessment. Young tissues and cells may respond well to such treatments; however, aged OPCs are impaired in their usual functions and it may be that they are unable to respond to such therapies. Given that the inability to remyelinate in MS patients is a function of age, and that these medications will probably be aimed at groups presenting progressive phases, it makes sense for such therapies to be tested on aged cells as well as animals before being taken to clinical trials.

There are numerous negative regulators of remyelination in the lesion environment. One of the most studied so far is the leucine-rich repeat and immunoglobulin-like domain-containing nogo receptor-interacting protein 1 (LINGO1), which is specifically expressed in the CNS. LINGO1 is well known as a regulator of axon outgrowth via the Nogo receptor, but has also been identified as a negative regulator of OPC differentiation (Mi et al., 2005). Inhibition of LINGO1 with antagonists resulted in OPC maturation *in vitro* and anti-LINGO1 antibodies promoted remyelination in both EAE and toxin-models of demyelination (Mi et al., 2009). Antagonising LINGO1 has been the main aim of RENEW (NCT01721161), a clini-

cal trial involving the human monoclonal anti-LINGO1 antibody opicinumab, however there was no difference in remyelination between the opicinumab and control group (Cadavid et al., 2017). A follow-up extension study (RENEWed (NCT02657915)) aims to assess the patients from the first trial for long-term effects on remyelination, however, very little is known about the relevance of LINGO1 in MS lesions and how the effects of LINGO1 are exerted in the animal models.

Wnt proteins are a family of secreted signalling molecules which canonically stimulate the transcriptional activity of β-catenin. Canonical Wnt signalling occurs in both developmental myelination and remyelination, however if the signalling pathway is dysregulated by expressing a constitutively active form of β-catenin in oligodendrocyte lineage cells, then both myelination and remyelination are impaired (Fancy et al., 2009). Wnt has been shown to act through the transcription factor Tcf4, which is highly expressed in active MS lesions, further suggesting its relevance in the disorder (Fancy et al., 2009). One of the strategies suggested to reduce and control Wnt signalling is the manipulation of one of its transcriptional targets, Axin2, shown to be required in OPC differentiation (Fancy et al., 2011). When expressed, Axin2 regulates Wnt signalling via a negative feedback loop, and its manipulation via the small molecule XAV939 stabilised Axin2 levels and accelerated remyelination (Fancy et al., 2011).

Notch is a transmembranous protein which acts as a receptor for Jagged1. Upon this interaction the Notch intracellular domain (NICD) is cleaved and translocated to the nucleus where it acts as a transcription factor (Schweisguth, 2004). Notch signalling inhibits OPC differentiation during development (Wang et al., 1998), however its involvement in remyelination remains uncertain (Stidworthy et al., 2004). Ablation of *Notch1* in early OPCs using Cre-*lox* via the *Olig1* promoter resulted in earlier OPC differentiation but had no impact on the overall rate of remyelination (Zhang et al., 2009). *Notch1* ablation in immature oligodendrocytes using the *Plp* promoter did not result in neither premature oligodendrocyte maturation, nor in accelerated remyelination (Stidworthy et al., 2004). The Jagged1-Notch pathway seems to be expressed in MS lesions, a downstream target of which is Hes5, an inhibitor of OPC differentiation (John et al., 2002), making this pathway a relevant, but not a dominant, candidate for negatively impacting OPC differentiation.

More recently, components of the extracellular matrix (ECM) have been implicated in remyelination failure. Of the many components making up the ECM, hyaluronan and chondroitin sulfate proteoglycans (CSPGs) have been implicated in OPC differentiation. CSPGs have been shown to prevent *in vitro* OPC maturation as well as their migration, and the use of fluorosamine, an inhibitor of CSPG synthesis, resulted in accelerated OPC recruitment and improved remyelination in the lysolecithin model (Lau et al., 2012; Keough et al., 2016). I

have previously discussed the involvement of regulatory molecules such as semaphorins in remyelination models and MS lesions; these are also valid candidates for future studies on potential targets in remyelination therapies.

Age is a critical factor in determining disease progression in patients affected by MS. This is partly due to aged microglia and macrophages being unable to phagocytose myelin debris, ultimately preventing OPC differentiation. *In vitro* studies and microarray analysis carried out by Natrajan et al. (2015) revealed that the aged phenotype of both rodent and human monocytes is in part due to an altered expression of Retinoid X receptor α (RXRα). The aged phenotype can be successfully obtained in young monocytes via a monocyte specific knockout of *RXR*α, whilst rejuvenation of old monocytes is possible via the use of RXR agonists. Furthermore, the ability of monocytes derived from aged rodents, aged humans and MS patients to phagocytose myelin debris increased upon their *in vitro* treatment with the RXR synthetic ligands 9-*cis*-retinoic acid (9cRA) and bexarotene. Modulation of phagocytosis via bexarotene treatment has also shown to increase the clearance of amyloid-β deposits in a mouse model of Alzheimer's Disease (Cramer et al., 2012). These findings are of the utmost relevance to MS patients, as bexarotene is an already FDA-approved rexinoid currently used for the treatment of cutaneous T cell lymphoma (Querfeld et al., 2006). Despite its relevance in monocytes, the effects of RXR are not limited to the immune system alone. RXRs are also expressed in oligodendrocyte lineage cells, and their role in remyelination was first described during a microarray analysis of the CNS toxin lesion (Huang et al., 2010a). It was revealed that the expression of RXRγ is highly increased at time points coinciding with the remyelination phase, and subsequent analysis of MS post mortem tissue suggested a correlation between RXRγ expression and remyelination capacity. Chronic inactive lesions contained significantly less RXRγ^+ oligodendrocyte lineage cells compared to active and remyelinated lesions. Huang et al. (2010a) went on to show that 9cRA treatment of *in vitro* OPC cultures and cerebellar slices induces OPC differentiation and *ex vivo* myelination, whilst the opposite is observed when RXRγ function is impaired via genetic and transcriptional silencing or its antagonists. Furthermore, 9cRA treatment of toxin-induced demyelination in aged rats resulted in accelerated remyelination (Huang et al., 2010a), and this work is the basis of an ongoing clinical trial that uses bexarotene, an RXR agonist, in MS patients.

In conclusion, in recent years numerous promising targets have been identified as candidates for the development of remyelination therapies, however, often the ageing component of MS is overlooked when testing their relevance in remyelination. All in all, RXRs are emerging as particularly interesting candidates for the above, as their activation can drive

OPC differentiation and remyelination in spite of ageing. However, the exact molecular mechanisms involved in RXR driven OPC differentiation remain elusive.

1.5 RXR belongs to the nuclear receptor superfamily

Nuclear receptors (NR) are ligand-induced transcription factors able to translate small chemical changes into large physiological effects by regulating gene transcription. They are ubiquitously expressed and are able to influence a vast range of tissue physiologies including development, growth, apoptosis and various aspects of homeostasis and metabolism (Evans and Mangelsdorf, 2014). Up until the 1960s, it had been extensively observed that small lipophilic molecules such as hormones and vitamins could influence tissue physiologies and induce *de novo* synthesis of mRNA (Edelman, 1975). How exactly these molecules exerted their function remained unknown until the development of radio-labelled oestrogen, which revealed the existence of a putative receptor that could translocate from the cytoplasm to the nucleus. The translocation was linked to the control of gene transcription (Jensen et al., 1966). This concept was further supported by the observation that metamorphic hormones in drosophila induced chromosomal puffing at specific locations, further implying a direct effect of these receptors on DNA (Ashburner et al., 1974). The nature of the putative receptor remained elusive until the mid-1980s, when the complementary DNA (cDNA) of the GCR and ER was isolated and cloned (Hollenberg et al., 1985; Miesfeld et al., 1986; Green et al., 1986). The characterisation of the first NRs and the observation of highly conserved homology at other genetic locations revealed the existence of a whole class of such receptors. For example, Green et al. (1986) reported that the ER shared extensive homology with the erb-A gene which was later discovered to encode a THR isoform (Weinberger et al., 1986). During these years the continuous discovery of highly homologous receptors activated by a whole range of specific lipophilic ligands revealed an array of new molecular pathways governing gene transcription.

1.5.1 Characterisation of the NR superfamily

NRs make up one of largest families of transcription factors, with over 270 NRs found in the nematode worm to much lower numbers in mammals including 47, 48 and 49 in rat, human and mouse respectively (Zhang et al., 2004; Evans and Mangelsdorf, 2014). With the use of gene sequence analysis it has been possible to track the emergence of the first NRs in early metazoans and the highly conserved evolutionary template that emerged from sequence comparison of NRs suggests they evolved from a common ancestor. Although Zn-fingers

with NR similarities are found in yeast and plants, we still lack the evidence for these to be considered their ancestors (Owen and Zelent, 2000). NRs emerged via two waves of gene duplication: the first occurred in the early metazoans and it divided NRs into subfamilies, whilst the second wave in vertebrates allowed the emergence of different isoforms of the same receptor (Szanto et al., 2004). The study of the evolutionary conservation and relationship of NRs characterised both structural and functional features that would allow the subsequent classification of the superfamily. Laudet (1997) divided the superfamily into six subfamilies as shown in Table 1.1.

The division in these subclasses remains arbitrary, and occasionally it is preferred to divide them into three subclasses depending on the types of ligands involved in their activation.

In this case, the first class is the 'steroid receptor family', and is broadly made of the NRs in class III of Table 1.1; the second class is the 'thyroid/retinoid family' made of mainly class I; and the final class is the 'orphan receptor family', for which the cognate ligands are still unknown (Bain et al., 2007). Ligands for some orphan NRs have been already described, including bile acids, fatty acids, cholesterol derivatives and so on, making them a particularly interesting avenue for the discovery of new signalling molecules (Chawla et al., 2001; Burris et al., 2013).

The NR structure resembles the one of other transcription factors in that it is divided into modules that correspond to autonomous functional domains as represented in Figure 1.5. Each domain is designed exquisitely for the specific signalling carried out by NRs. NRs are made of six modular domains labeled A to F. The A/B domain is located at the N-terminal of the receptor and is the least conserved region. In fact, alternative splicing of this module accounts for the creation of different isoforms of the same receptor. This domain is subject to post-translational modifications such as phosphorylation, which can influence their transcriptional activity, as in the case of RAR, PPAR and ER, which can be phosphorylated by cyclins and MAPK (Kato et al., 1995; Rochette-Egly et al., 1997, 2000; Juge-Aubry et al., 1999). This is because the A/B module contains an autonomous transcriptional activation function termed AF-1, responsible for the constitutive ligand-independent activation of the receptor (Tora et al., 1989; Tzukerman et al., 1994; Burris et al., 2013). The C module is the most conserved and contains the DNA binding domain (DBD), which includes two Zn-finger core motifs that allow NRs to associate to the genome (Lee et al., 1993; Schwabe et al., 1993; Mader et al., 1993). The DBD is followed by a hinge region (D module), conveying flexibility to the DBD and allowing rotation of the module. The hinge region is between the DBD and the ligand binding domain (LBD), termed E module (Aranda and Pascual, 2001). The main function of the LBD is to allow binding of the ligand and contains AF-2, the ligand-

Class	Receptor	Abbreviation	Isoforms	Ligand
I	Constitutive androstane receptor	CAR	α,β	Androstanes
	Farnesoid X receptor	FXR		Bile acids
	Liver X receptor	LXR	α,β	Oxysterols
	Peroxisome proliferator activated receptor	PPAR	α,β,γ	Eicosanoids, Thiazolidines
	Pregnane X receptor	PXR		Pregnanes
	Retinoic acid receptor	RAR	α,β,γ	Retinoic acid
	Reverse ErbA	RevErb	α,β	Unknown
	Retinoid Z receptor	RZR	α,β,γ	Unknown
	Thyroid hormone receptor	THR	α,β	Thyroid hormone
	Vitamin D receptor	VDR		Calcitriol
II	Chicken ovalbumin upstream promoter transcription factor	COUP-TF	α,β,γ	Unknown
	Hepatocyte nuclear factor 4	HNF-4	α,β,γ	Fatty acyl-CoA thioesters
	Photoreceptor-specific nuclear receptor	PNR		Unknown
	Retinoid X receptor	RXR	α,β,γ	9cRA
	Tailles-related receptor	TLX		Unknown
	Ubiquitous receptor	UR		Unknown
III	Androgen receptor	AR		Androgens
	Oestrogen receptor	ER	α,β	Oestradiol
	Oestrogen-related receptor	ERR	α,β,γ	Unknown
	Glucocorticoid receptor	GCR		Glucocorticoids
	Progesterone receptor	PR		Progestins
	Testis receptor	TR2	α,β	Unknown
IV	NGF-induced clone B	NGFI-B	α,β,γ	Unknown
V	Steroidogenic factor 1	SF-1	α,β	Oxysterols
VI	Germ cell nuclear factor	GCNF		Unknown

Table 1.1 The nuclear receptor superfamily is divided into six subfamilies based on their amino acid sequence conservation. The full name and abbreviation of the receptors, known subtypes and ligands are shown. Table adapted from Aranda and Pascual (2001).

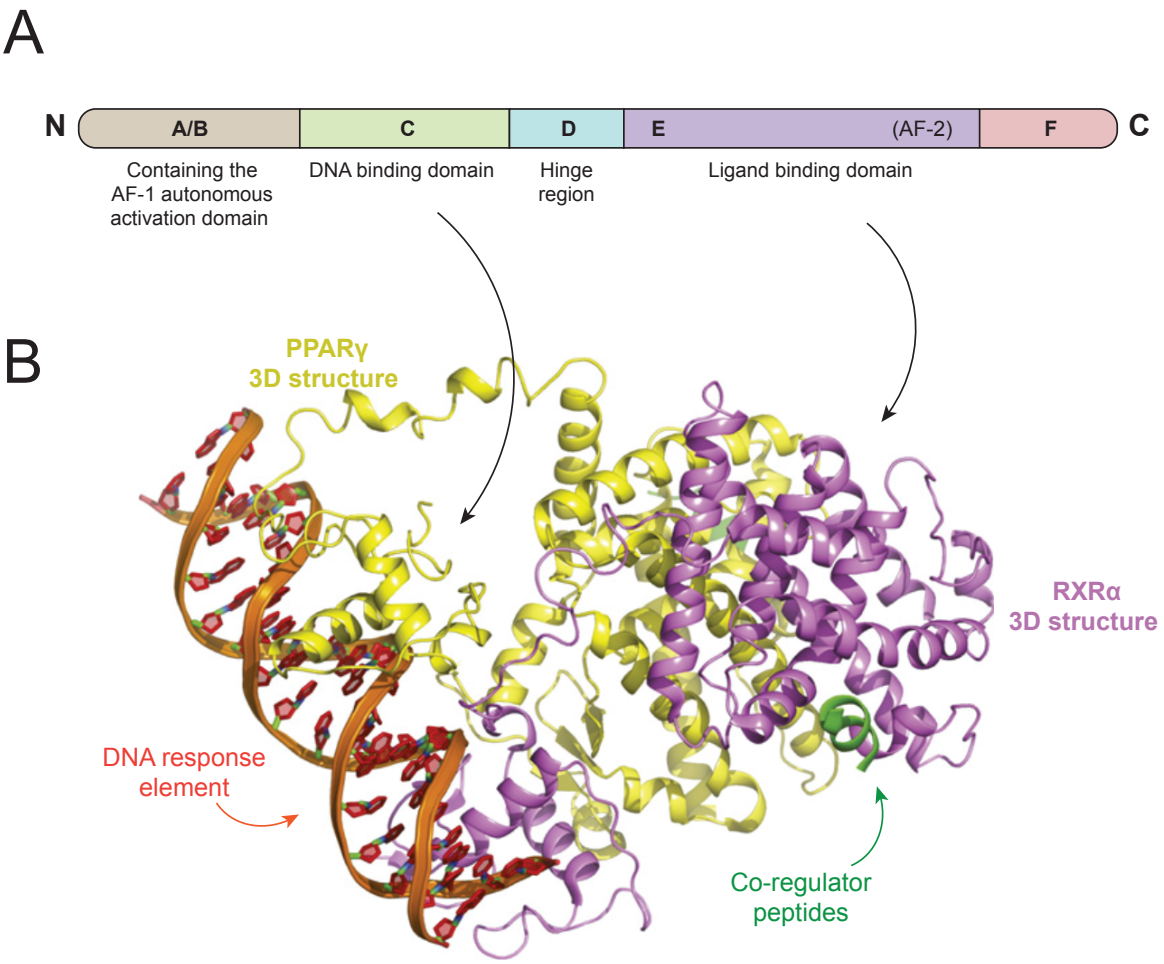


Fig. 1.5 Schematic of NR structure. (A) is a diagram representing the modular structure of a generic NR. It is divided into autonomous domains. The A/B domain is located at the N-terminal. This region also contains the ligand-independent transactivation domain AF-1. The C domain binds to genomic elements. The D domain is a hinge region that allows for flexibility. The E domain is involved in ligand binding and contains the ligand-dependent transactivation domain AF-2. The role of the F region remains unknown. (B) illustrates the 3D quaternary structure of the RXRα-PPARγ heterodimer, where RXRα is illustrated in pink and PPARγ is shown in yellow. The complex is shown bound to DNA, represented in orange. Co-regulator peptides associated to the heterodimer are shown in green. This figure was adapted from Kojetin and Burris (2013) and their 3D quaternary structure diagram was used.

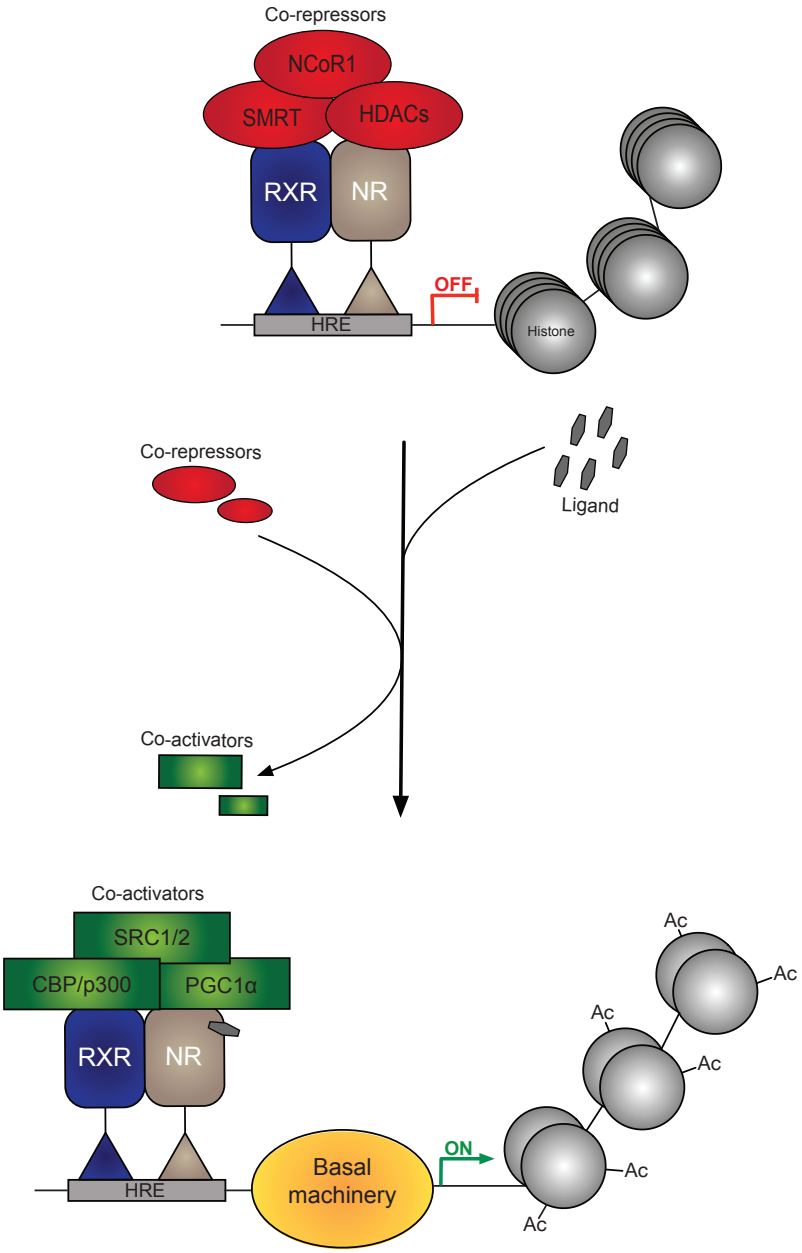


Fig. 1.6 Schematic representation of the signalling mechanism of RXR heterodimers. It is generally accepted that in the absence of their cognate ligand the RXR-NR dimer is bound to relevant HREs and associated to co-repressors inhibiting gene transcription. In the presence of the ligand co-repressors are released and co-activators bind instead. The co-activators allow for chromatin modifications and remodelling, and with recruitment of the basal machinery gene transcription can now occur.

dependent transactivation domain (Wurtz et al., 1996; Nagy and Schwabe, 2004). However, the LBD exerts multiple functions that go beyond containing the ligand binding pocket of NRs. It also mediates binding of heat-shock proteins and dimerisation of the receptors (Pratt et al., 1988; Scherrer et al., 1993; Valentine et al., 2000).

1.5.2 RXR heterodimers are central to NR signalling

The ability of NRs to regulate gene transcription is determined by genomic variants of the core motif AGGTCA, which allow sequence-specific binding to the genome (Beato et al., 1995; Evans and Mangelsdorf, 2014) . These core consensus sequences are termed hormone response elements (HRE) and are usually located in upstream regulatory promoter or enhancer regions of genes (Kittler et al., 2013; Gadaleta and Magnani, 2014). Differences in the orientation and spacing of the HREs determine how NRs associate to the DNA, revealing that NRs can associate to the genome as homodimers or heterodimers as well as monomers. Class III NRs tend to associate to certain palindromic repeats as homodimers, whilst the non-steroidal class I NRs heterodimerise with RXR when associated to the DNA (Beato, 1991; Yu et al., 1991; Bugge et al., 1992). RXR is capable of heterodimerisation, and this finding gave rise to a wave of discovery that shifted the vision of NR signalling from individual linear signalling pathways to the astonishing realisation of the existence of novel interlaced signalling networks, developing new and fundamental concepts in the NR field.

It is conventionally accepted that unlike steroid NRs, which shuttle to the nucleus in presence of the ligand, RXR heterodimers are already found in the nucleus associated to their relevant HRE. However recent studies have shown that shuttling does occur for RXR heterodimers as well. In the absence of their ligand, they are also associated to co-repressors which prevent transcription of the downstream gene. Being small lipophilic molecules, NR ligands can cross plasma membranes, and in the presence of their cognate ligand the RXR-NR will release the repressors, co-activators will bind in their place, leading to chromatin modifications that will make the chromatin more accessible to the basal machinery. Once this is recruited, transcription of the downstream genes can occur.

However, the opportunity of discovering new signalling avenues via heterodimeric signalling comes at the cost of an increasingly complex system to disentangle where, the more is learnt, the bigger the realisation of how little is known. The above remains an extremely simplistic description as we still lack knowledge on the complexity of regulation of RXR-NR signalling, but what does regulation of this system actually entail? As summarised in Figure 1.7, for RXR-NR to signal, four main things need to happen, suggesting that the system can be regulated at one or more of the following levels.

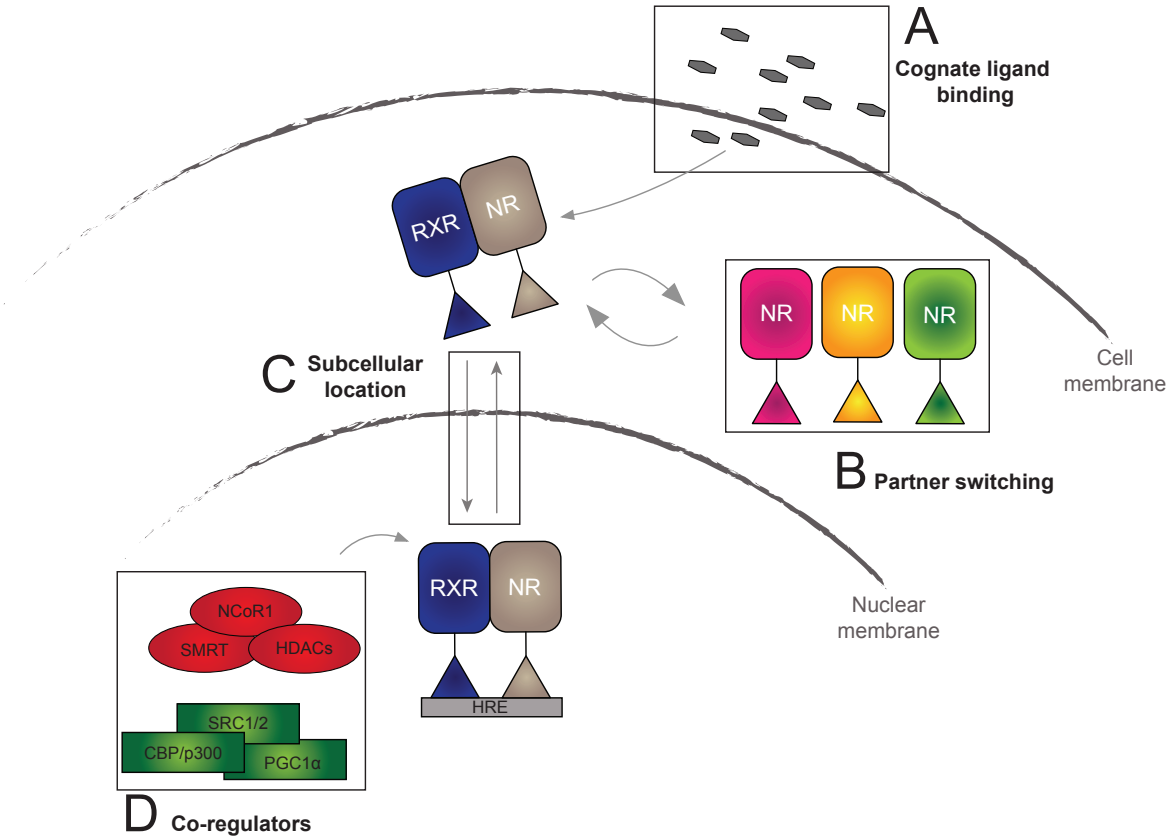


Fig. 1.7 Schematic of the levels of control for NR signalling. In order for RXR heterodimers to signal four main things need to happen: (A) firstly the cognate ligand needs to be present. The ligands are small lipophilic molecules that can cross cellular membranes without the need of transporters. (B) Upon ligand binding, RXR heterodimerises with the correct NR partner. This is essential for both shuttling and association to the desired HREs. (C) Although it is conventionally accepted that RXR heterodimers are always found in the nucleus, it has been shown that whole heterodimers are able to shuttle between the cytoplasm and nucleus upon ligand binding. Subcellular location is essential to the signalling of these transcription factors and segregation in specific cellular compartments is thought to be one of the ways in which this system is regulated. (D) Upon HRE binding co-regulators are recruited in order to modify the chromatin and control transcription of the desired genes. Depending on the HRE element bound, either co-activators or co-repressors are recruited which may promote or inhibit gene transcription.

Cognate ligand binding

NRs are one of the most commonly used drug targets for both human disease and lifestyle (Overington et al., 2006). Despite being mainly studied and used in the cancer field, NRs have also been targeted in metabolic and inflammatory disorders such as diabetes and rheumatoid arthritis as well as for the contraceptive pill and performance enhancing steroids (Overington et al., 2006; Wang et al., 2010; Shirinsky and Shirinsky, 2011; Sladek, 2011; Dhiman et al., 2018). This is due to their hydrophobic binding pocket, which permits easy manipulation of their biological function with the use of both natural and synthetic ligands. As illustrated in Table 1.1, NR ligands are small hydrophobic molecules including lipophilic hormones, vitamins, xenobiotics, derivatives of retinoids, fatty acids, cholesterol and many more. In the absence of the cognate agonist, the RXR-NR is in a conformation that allows association of co-repressors which will silence transcriptional activity (Casanova et al., 1994; Tong et al., 1996). When the agonist is present, it binds to hydrophobic signatures of the pocket via van der Waal interactions (Huang et al., 2010b). X-ray crystal structure of NRs revealed that this causes a structural conformational change of the receptor that releases the repressors and exposes a hydrophobic surface which will bind co-activators possessing the corresponding binding motif (Heery et al., 1997; Nolte et al., 1998; Gronemeyer et al., 2004).

The above is an extremely simple outline of the general consensus of how ligands affect NR signalling and is based on the studies of the first NRs. The idea of a single ligand for a single receptor which will elicit a very specific biological outcome remains an oversimplification that is not typical of all NRs, especially RXR heterodimers. Firstly, we find dual ligand regulation, where two different ligands are able to induce different outcomes by activating the same receptor complex; this will be further discussed in the next section. It has also been shown that many of these receptors can be activated by a variety of molecules rather than by single ligands. This depends on the binding pocket of the individual receptors, which could accommodate varieties of similarly structured molecules, and in some instances ligands can be interchangeable as in the case of COUP-TF, whose crystal structure was found to contain retinoic acid in its pocket and is also able to respond to it in transactivation assays (Kruse et al., 2008). Adding to this complexity, crystal structure of the RXR partner PPAR revealed two molecules bound simultaneously in the pocket of the receptor, with different residues mediating the recognition and binding of different ligands (Tsukahara et al., 2006; Bernardes et al., 2013). This final observation shows that unlike class III NRs, which are typically viewed as endocrine receptors, RXR-heterodimers act as environmental sensors and the overall biological outcome of their stimulation may be a result of the environmental cocktail of ligands at that specific point in time. Finally, an important ability of NRs is that of regulating their own activation by controlling the transcription of cytochrome P450 (CYP)

enzymes involved in the production or degradation of their ligands. This has been described for RXR, VDR, RAR, PPAR, PXR, LXR and FXR, and is important as these dynamics are the drivers of physiological circuits (Kliewer et al., 1998; Wang et al., 1999; Evans and Mangelsdorf, 2014).

Heterodimerisation of the correct NR partner

The dual ligand regulation of RXR heterodimers is one of the most fascinating aspects of their biology, as the same receptor complex can be activated by two different ligands, one acting on RXR and one acting on the partner. This ability divides RXR heterodimers into two categories: non-permissive and permissive. Non-permissive heterodimers are formed by RXR dimerising with RAR, THR and VDR. The ligand-dependent activity of RXR in these complexes is suppressed by the associated partner, so the heterodimer can only be activated in the presence of the partner's ligand (Leblanc and Stunnenberg, 1995). The study of the non-permissive heterodimer RXR-THR via nuclear magnetic resonance spectroscopy (NMR) revealed that liganded THR induces a conformational structure where the LBD of RXR is physically blocked, preventing the entrance of the RXR ligand (Kojetin et al., 2015). Statistical coupling analysis predicts this mechanism to be conserved across non-permissive partners (Shulman et al., 2004). In this scenario, RXR has often been termed the 'silent partner', believed to play an auxiliary role in stabilising the complex for the signalling of its non-permissive partners. However, recent data revealed that the presence of rexinoids modulates the interaction of RXR-VDR and RXR-THR with co-regulators, suggesting a more dynamic allosteric signalling pathway than anticipated (Sánchez-Martínez et al., 2006; Fattori et al., 2015). Permissive RXR partners include FXR, LXR, PXR, and PPAR, and when bound to these the RXR-NR can be activated by the ligand of either receptors (Evans and Mangelsdorf, 2014). If both ligands bind simultaneously, they act synergistically as shown in the case of RXR-PPAR, where the net effect of the combined ligands is stronger than with individual ligands (Kliewer et al., 1992). It therefore appears that the overall biological effect of the RXR-NR activation may not be dictated exclusively by the presence of the ligand, as the ability of ligands to activate the heterodimers reciprocally depends on structural changes on RXR induced by the partner itself.

All tissues express multiple RXR partners simultaneously, begging the question of how do certain RXR heterodimers prevail over others, if at all. If we therefore consider a limited pool of available RXR at any one time, we can imagine the rise of a competitive system where the availability/sequestration of RXR is dependent on the expression level of all of its partners. This hypothesis is supported by the observation of extensive cross-talk between RXR and its partners as well as between the partners themselves (Chan and Wells, 2009). An

example is PPAR, which has been found to compete with VDR for its binding with RXRα. In this study, overexpression of PPARγ abrogated the effects of calcitriol activated VDR, which could in turn be rescued by overexpressing RXRα, suggesting the competitive nature of these receptors (Alimirah et al., 2012). The above has also been reported between PPAR, THR and LXR (Hunter et al., 1996; Ide et al., 2003). Further regulation of the system is therefore achieved with RXR availability as a limiting factor for NR signalling.

Shuttling to the nucleus

Although ligand availability and partner association both play important roles in regulating RXR-NR activity, other mechanisms have been hypothesised to contribute to the responses modulated by RXR heterodimers. An example is the subcellular compartmentalisation of the dimers. As NRs have been shown to continually shuttle between the nucleus and cytoplasm of cells (DeFranco, 2002) this is a coherent hypothesis as to elicit transcriptional regulation, transcription factors need to be located in the nucleus. Active nuclear import and export of proteins is mediated by nuclear localisation sequences (NLS) in the protein to be transported. These are recognised by importins, which in turn mediate the nuclear import of the cargo by interacting with machinery at nuclear pores (Kumar et al., 2006). NRs harbour one or more types of NLS, therefore suggesting that the nuclear translocation of such receptors is a regulated event (Hsieh et al., 1998; Black et al., 2001; Prüfer and Barsony, 2002; Zhang et al., 2018). The use of fluorescent protein-labelled chimeras and fluorescence resonance energy transfer microscopy (FRET) has aided many of the discoveries made regarding the translocation of RXR-NR between the nucleus and the cytoplasm. If we take RXR-VDR as an example, both receptors can be transported into the nucleus as monomers via two distinct mechanisms. However, RXR dimerises with VDR in the cytoplasm and can be translocated to the nucleus as an already formed heterodimer (Prüfer et al., 2000; Yasmin et al., 2005). FRET and photobleaching experiments suggest that the subcellular distribution of liganded and unliganded VDR is highly dependent on RXR, and heterodimers containing mutant RXR failed to distribute unliganded VDR. This correlated with reduced basal transcriptional activity (Prüfer et al., 2000). However, in response to the VDR ligand calcitriol, RXR-VDR shuttles to the nucleus. This effect is not obtained in the presence of 9cRA, nor by mutation of the RXR NLS, suggesting that, for this heterodimer, the partner dominates the localisation and consequently the activity of the receptor complex (Prüfer and Barsony, 2002; Yasmin et al., 2005). Therefore, the distribution of both the heterodimers and monomers is another important level of controlling their signalling pathway.

Association of co-regulators

The ultimate function of RXR heterodimers is to regulate gene transcription, and this is dependent on the recruitment of factor complexes that are involved in determining chromatin state and therefore its accessibility. A generally accepted view is that in absence of the ligand, RXR-NR binds to co-repressors via a specific motif, able to interact with the LBD, consequently silencing transcriptional activity (Casanova et al., 1994; Tong et al., 1996; Perissi et al., 1999; Webb et al., 2000). However, the conformational change imposed by ligand binding releases co-repressors and exposes a hydrophobic surface to which co-activators can now associate and induce chromatin modifications that will allow transcription (Egea et al., 2000; Evans and Mangelsdorf, 2014). Examples of co-repressor molecules include silencing mediator for retinoic and thyroid hormone receptor (SMRT) and nuclear co-repressor (NCoR), both of which have been shown to bind RAR and THR *in vitro*, however are released upon treatment with the respective agonists (Hörlein et al., 1995; Chen and Evans, 1995). These two complexes are known to directly interact with HDACs (Kao et al., 2000; Huang et al., 2000), suggesting that one of the mechanisms by which SMRT and NCoR prevent gene transcription is by inducing histone de-acetylation via HDACs. Common co-activator complexes include ATP-dependent chromatin remodeller factor SWI/SNF, HATs such as CBP/p300 and the TRAP/DRIP complex, which is involved in the recruitment of RNA polymerase II (Dilworth et al., 2000; Rachez et al., 2000; Ito et al., 2002).

Although the above view is still generally accepted in the field, recent research has clearly shown that the above remains a overly simplified model of how these receptors control gene transcription. Co-regulator recruitment is highly dependent on the NR structure imposed by the presence and sequence of its HRE (Fernandez, 2018). This has been shown, for example, in biophysical studies involving RXR-THR and RXR-VDR (Putcha and Fernandez, 2009; Zhang et al., 2011). In the former study it was observed that the binding affinity of the co-activator SRC1 is altered in the presence or absence of relevant HREs (Putcha and Fernandez, 2009), whilst the latter study revealed that the binding affinity alterations between co-regulators and RXR-VDR are due to the structural dynamicity caused upon DNA binding (Zhang et al., 2011). Furthermore, the composition of the HRE is also able to induce the inverse effect of what is expected from a NR agonist, therefore, upon binding of liganded RXR-NR, co-repressors are recruited and transcription is inhibited. (Fernandez, 2018). These are termed negative HREs and have been described for numerous RXR partners as well as class III NRs (Schoorlemmer et al., 1994; Radoja et al., 1997; Koszewski et al., 2000; Dostert and Heinzel, 2004; Sharma et al., 2013).

Thus, RXR is an integral part of NR signalling as it can form heterodimers with other NRs. Signalling of RXR heterodimers is controlled at different molecular levels, whereby each level influences the other. Heterodimerisation as well as nuclear import/export both depend on ligand presence and are partner dependent processes. The co-regulator association is further dependent on ligand binding and DNA interactions, both of which have long-range effects on the heterodimer structure, and it is this allosteric communication between ligand, DNA and co-regulator, that provides a link between the target genomic area, the distinct co-regulator recruited and the consequent chromatin remodelling that will occur (Fernandez, 2018).

1.6 RXR and its partners influence aspects of OPC biology important to remyelination

1.6.1 The involvement of RXR and its partners in remyelination

Despite being highly conserved and ubiquitously expressed, it is clear that RXR heterodimers carry out process-specific functions. One of these is their involvement in regulating stem cells, as they are key players in their self-renewal as well as in their differentiation and re-programming (Sun and Shi, 2010). These aspects also hint at their potential as an important avenue for regenerative medicine. Indeed, NRs have been found to regulate regenerative processes in a variety of organs, including skin, bone and liver, and have been found to influence both the early inflammatory stage of regeneration as well as the later reparative phase (Vacca et al., 2014; Jin et al., 2015; Rieger et al., 2015; Merlen et al., 2017). In this regard, remyelination is no exception.

Although the impact of NRs on the immune system will not be discussed further, it is worth mentioning that EAE mice treated with agonists for THR, VDR, PPAR etc. result in lower clinical score and amelioration of the symptoms, a result which is compromised when carrying out the same experiments in the respective NR knockdown or knockout animals. Further *in vivo* and *in vitro* assessments suggest that these ligands modulate the immune system of EAE animals in a variety of ways, including regulating phagocytic activity, reducing pro-inflammatory aspects such as CNS monocyte infiltration, activation and gliosis, whilst increasing the number of cells in an anti-inflammatory state (Calza et al., 2002; Lovett-Racke et al., 2004; Hindinger et al., 2006; D'Intino et al., 2011; Joshi et al., 2011; Kanakasabai et al., 2011; Mayne et al., 2011; Dell'Acqua et al., 2012; Paintlia et al., 2013; Montarolo et al., 2015;

Secor McVoy et al., 2015; Zhen et al., 2015). Whilst the immunomodulatory aspect of NR signalling in the above studies is of obvious relevance to the early clinical stages of MS, their influence on remyelination remains unclear. Instead, to address the above, a series of NR agonists and antagonists have been tested in toxin-induced demyelination models. In the cuprizone model, treatment with thyroid hormone has shown the most successful results. Thyroid hormone treated mice present an increase in differentiation markers such as MBP in both the CC and the cortex (Franco et al., 2008; Silvestroff et al., 2012). However, they also observed an increase in numbers of $Olig2^+$, suggesting increased proliferation (Franco et al., 2008). Enhanced OPC differentiation may therefore be the result of increased OPC numbers and density rather than a direct effect on differentiation. Despite this, remyelination assessment via EM showed that, unlike what has been observed in remyelinated tissue, upon thyroid hormone treatment a lower *g* ratio is observed compared to controls, suggesting that new myelin is similar to that of developmental myelin (Zhang et al., 2015a). Supplementation of vitamin D via dietary intake or intraperitoneal injections also yielded positive effects in the cuprizone model, whereby it showed a protective effect by reducing the extent of demyelination as well as increasing myelin content during the defined remyelination phases (Wergeland et al., 2011; Nystad et al., 2014). On the other hand, activation of LXR, RXR and RAR did not yield any differences in $CC1^+$ staining in the cuprizone model, suggesting they had no effect on OPC differentiation and consequently on remyelination (Kruczek et al., 2015).

The choice of the cuprizone model makes demyelination induction a less invasive procedure. However, it does come at a cost as a drawback of this model is the ambiguous separation between demyelination and remyelination, as both occur simultaneously. It may therefore be that these studies were undermined by the caveats of the cuprizone model which impeded correct analysis and interpretation of the data since, for example, Huang et al. (2010a) clearly showed the positive effects of RXR activation on remyelination in the EB model. These effects have been previously discussed in Section 1.4.4.

There is currently little data on the effects of RXR partners on *in vivo* remyelination, and the majority of the data has been collected from cuprizone animals, however NRs have been shown to have profound effects on OPC proliferation and differentiation both *in vitro* and *ex vivo*. Based on this data, I hypothesise that RXR and its partners can influence differentiation in three ways: by cell cycle control, by directly promoting differentiation and by aiding in fatty acid and cholesterol metabolism.

1.6.2 RXR and its partners regulate cell cycle and differentiation

The main field of research involving the study of NRs is the cancer field, as NRs play critical functions in both the development and progression of cancers (Dhiman et al., 2018). Additionally, they have been particularly useful for the development of cancer differentiation therapies. As discussed by Altucci et al. (2007), unlike conventional chemotherapy which aims at killing cancerous cells, cancer differentiation therapy tries to induce terminal differentiation/apoptosis of tumorous cells by reactivating pathways that are suppressed during tumorogenesis. RAR has been the most successful target for this treatment and is currently used for the treatment of acute promyelocytic leukaemia (APL), showing high remission rates (Cicconi and Lo-Coco, 2016). It has been currently shown that the induction of differentiation can also be obtained with the use of rexinoids, which are more favourable as are generally less toxic than retinoids (Miller et al., 1997).

The ability to induce differentiation and influence proliferative cells is not restricted solely to RAR and RXR, but is observed across the NR superfamily. Indeed, there is increasing evidence that NRs can regulate numerous aspects of stem cell biology, including maintenance of stemness, induction of differentiation and trans-differentiation, as well as being use in stem cell reprogramming (Jeong and Mangelsdorf, 2009). They do so for embryonic stem cells (ESC) as well as for adult stem cells. However, the way in which NRs influence stem cell regulation is tissue-specific, a surprising finding considering their highly conserved evolution.

RAR is a potent master regulator of embryonic development, which became clear via the studies of its teratogenic effects (Durston et al., 1989; Avantaggiato et al., 1996). In the bone marrow of RAR$\gamma^{-/-}$ mice, there is a reduction in the hematopoietic stem cell (HSC) pool and an increased number of differentiated cells, suggesting an inability in maintaining the HSC population (Purton et al., 2006). This is also observed in mesenchymal stem cells (MSC) where the use of RARα and β antagonists *in vitro* resulted in chondrogenesis (Kafienah et al., 2007). In the context of OPC biology, in the embryonic spinal cord retinoic acid (RA) prevents their maturation (Noll and Miller, 1994) independently of the stimulation of cell division (Laeng et al., 1994). RAR may elicit these effects by influencing the expression level of the negative regulators of differentiation *Hes5* and *Id4* as has been shown by recent RNA sequencing experiments, where OPCs derived from human ESCs were exposed to all-*trans* retinoic acid (ATRA) (Kim et al., 2017).

The role of thyroid hormone as a potent driver of OPC differentiation has been extensively described both *in vitro* and in the developmental *in vivo* setting. During development, animals with hypothyroidism due to lack of triiodothyronine (T3) present delayed OPC differentiation and myelination, whilst the opposite is observed in hyperthyroidic animals,

suggesting a link between thyroid hormone and myelination (Ibarrola and Rodríguez-Peña, 1997; Marta et al., 1998). Both THRα and β are expressed in OPCs, and the pattern of expression coincides with the peak of myelination in P7 rats. This time point coincides with a switch in the expression of the THR isoforms in OPCs, where THRβ is the prevalent isoform (Carré et al., 1998; Sarlieve et al., 2004). T3 potentiates oligodendrocyte lineage terminal differentiation *in vitro* (Almazan et al., 1985; Koper et al., 1986; Dugas et al., 2012) and its use has been extensively adopted in tissue culture upon wanting to differentiate OPCs into oligodendrocytes, as well as for the generation of oligodendrocytes from human iPSCs (Ehrlich et al., 2017). OPC differentiation in T3 presence occurs at the expense of proliferation as shown by the decrease of BrDU incorporation, a phenomenon observed both in the presence as well as the absence of PDGF and FGF-2, suggesting that THR activation overrides the effect of mitogens involved in maintaining OPC proliferation (Barres et al., 1994).

VDR signalling is involved in osteoclast formation of chondrocytes, suggesting an involvement in their terminal differentiation (Masuyama et al., 2006). Moreover, VDR is also shown to be involved in cell lineage specification in the CNS, where multipotent NSCs treated with vitamin D show both a more proliferative phenotype and acquire an oligodendrocyte fate rather than a neuronal one (Shirazi et al., 2015). However, OPCs treated *in vitro* with calcitriol differentiate into oligodendrocyte at the expense of proliferation, suggesting VDR as a potent regulator of cell cycle exit and differentiation induction (de la Fuente et al., 2015). In this study, de la Fuente et al. (2015) further showed that antagonising VDR signalling both *in vitro* and in *ex vivo* organotypic cerebellar slices blocked baseline differentiation and remyelination. These effects are linked to the RXR-VDR heterodimer based on co-immunoprecipitation (CoIP) experiments carried out on oligodendrocyte lineage cells (de la Fuente et al., 2015).

As well as in the toxin-induced lesion, Huang et al. (2010a) demonstrated the specific effects of RXRγ on OPCs both *in vitro* and in cerebellar slices. The transcriptional and pharmacological inhibition of RXRγ resulted in reduced differentiation and remyelination, whilst agonist activation increased both criteria. *RXRγ* knockout animals are apparently normal (Krezel et al., 1996), however, they do present depressive-like behaviours due to reduced expression levels in dopamine D2 receptor, altered serotonin levels (Krzyzosiak et al., 2010) and deficits in working memory, for which the molecular underpinning is still unknown but could be due to impaired long term potentiation and depression (Wietrzych et al., 2005; Chiang et al., 1998), and consequently synaptic plasticity. It is interesting to note that in CNS cells, RXRγ is predominantly expressed in OPCs as observed in the RNA-seq database generated by Zhang et al. (2014). Furthermore, it falls within the top 500 genes

differentially expressed in OPCs compared to newly formed and mature oligodendrocytes, further suggesting a salient role in these cells. It is clear that, based on *in vitro* data, all the above NRs influence different aspects of cell cycle and differentiation, and RXRγ may be involved in both areas, depending on which partners it predominantly associates to. This has led to an unproven model whereby RXRγ switches its principal binding partner as OPCs progress through the lineage, with each heterodimer being responsible for the maintenance of a stage or progression to the next (Franklin and ffrench Constant, 2017).

1.6.3 RXR partners aid in cholesterol and fatty acid metabolism

Differentiation is an energy demanding task due to the dramatic changes that progenitor cells need to undergo transcriptionally and metabolically (Folmes and Terzic, 2014). PPAR is critical in energy and lipid homeostasis, regulating metabolic function based on the control of genes involved in both lipid uptake and storage. Due to their clear metabolic function, PPARs have been used extensively in the treatment of metabolically derived disorders, such as obesity and type II diabetes (Choi et al., 2014). PPARβ and γ both play a role in OPC differentiation *in vitro*, as their activation results in increased MBP and PLP expression, and pushes glial progenitors into acquiring an oligodendrocyte fate (Granneman et al., 1998; Saluja et al., 2001; Sim et al., 2008). The differentiation observed could be due to increased metabolic support by PPAR activation, as the use of both natural and synthetic PPAR ligands on primary OPCs resulted in increased mitochondrial activity as well as promoting antioxidant protective effects via enzymes involved in protection against reactive oxygen species (Bernardo et al., 2009; De Nuccio et al., 2011, 2015; Bernardo et al., 2017).

Another RXR partner involved in fatty acid metabolism is LXR. LXRβ is expressed in the oligodendrocyte lineage, and general LXR$\gamma^{-/-}$ mice present a higher *g* ratio and reduced expression of oligodendrocyte markers compared to wild type animals (Makoukji et al., 2011; Meffre et al., 2015). Although it cannot be excluded that the above could be a consequence of compromised neurons rather than a defect in myelin *per se* (Wang et al., 2002), force-feeding wild type controls with the LXR agonist T0901317 (T090) increases MBP and PLP expression both at the transcriptional and protein levels, as well as promoting *in vitro* OPC differentiation and remyelination in cerebellar slices (Meffre et al., 2015). LXRs are key players in the regulation of cholesterol homeostasis (Courtney and Landreth, 2016a). The brain contains a quarter of total body cholesterol, which is produced locally in the CNS. Within the CNS, myelin has the highest cholesterol content (Vitali et al., 2014; Courtney and Landreth, 2016a). Saher et al. (2005) have shown the importance of cholesterol in oligodendrocyte myelination with the use of mice in which the cholesterol biosynthesis pathway was selectively inhibited in CNP^{+} by the impairment of squalene synthase.

These mice present with severe phenotypes such as ataxia, tremors and premature death, and although oligodendrocytes appeared healthy, there was severe CNS hypomyelination (Saher et al., 2005). Oligodendrocyte cholesterol availability is fundamental, and regulators of cholesterol metabolism such as LXR have critical roles in supporting OPCs in their maturation towards becoming mature oligodendrocytes.

Thus, RXRγ is a positive regulator of OPC differentiation and remyelination. Its partners have been involved in important aspects of OPC biology, such as cell cycle progression, promoting differentiation or providing metabolic support to differentiating OPCs. These effects elicited by the partners may involve RXRγ heterodimers, leading to the development of the unproven 'partner switching' model.

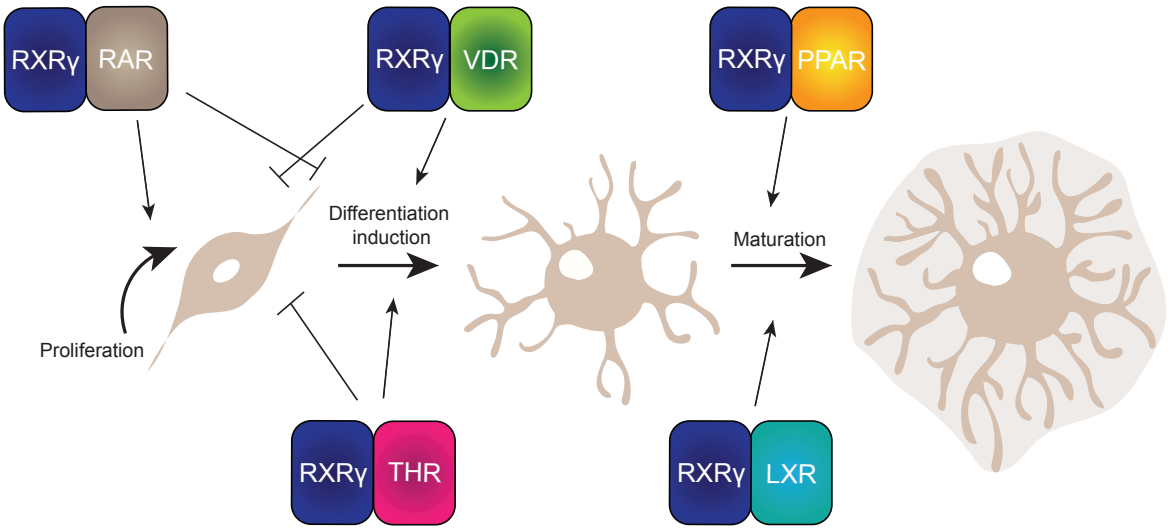


Fig. 1.8 Schematic of the partner switching model. In this model it is hypothesised that RXRγ acts as an anchor protein and switches its principal binding partner as OPCs progress through the lineage. This is because each heterodimer is responsible for the maintenance of a lineage stage or progression to the next. This is based on *in vitro* and *in vivo* data whereby RAR activation maintains OPCs in their proliferative state. On the other hand, activation of VDR or THR promotes OPC differentiation at the expense of proliferation, suggesting their involvement in inducing the first stages of differentiation. PPAR and LXR activation have also been involved in OPC differentiation. Due to their involvement in fatty acid and cholesterol metabolism as well as on *in vivo* data, they may be involved in the terminal stages of differentiation where oligodendrocyte maturation and myelin sheath formation occur.

1.7 Aims and objectives

RXRγ, as well as its partners, influence various aspects of OPC biology relevant to its regenerative potential. However, how the effects observed by the partners are elicited via RXR heterodimerisation remains unknown.

Due to the pleiotropic signalling of RXR, this information will aid in the development of specific remyelination therapies targeting appropriate heterodimers and reducing treatment side effects.

I hypothesise that RXRγ acts as an anchor protein for the association of different NRs, and that different RXRγ heterodimers regulate different steps of oligodendrocyte lineage progression.

Furthermore, RXRγ acts by controlling transcription of target genes, and I hypothesise these to be involved in oligodendrocyte-specific differentiation.

To answer these hypotheses I have set out to:

- Identify RXRγ binding partners important in the oligodendrocyte lineage;
- Understand which heterodimers are important at different stages of lineage progression;
- Understand whether the system is regulated by other NR signalling levels such as ligand, subcellular localisation and co-regulator binding;
- Identify the genes controlled by RXRγ in oligodendrocyte lineage cells;
- Understand how OPC ageing affects RXRγ and its partners.

Chapter 2

Materials & Methods

2.1 Materials

A table of all materials and kits used can be found in Appendix A, Table A.1.

2.2 Methods

2.2.1 Animal husbandry

All animal studies were ethically reviewed and carried out in accordance with the Animals Scientific Procedures Act of 1986 and the GSK Policy on the Care, Welfare and Treatment of Animals. The rat strain used for this project was Sprague Dawley (SD). SD rats were bred in the Innes and MIRA Animal Facilities at the University of Cambridge. All animals were kept under a light/dark cycle of 12 hours and fed a standard choke diet.

2.2.2 OPC isolation via mixed glia culture

Postnatal day 0-3 SD rats were euthanised via an overdose of intraperitoneal Pentoject injection following the Schedule 1 procedures of the Animal Scientific Procedures Act of 1986. The brains were removed from the skulls and transferred to a petri dish of ice-cold Hibernate A for low fluorescence (HALF), for which the formulation is shown in Table 2.1. The brains were dissected by removing the meninges, olfactory bulbs, hindbrain and midbrain. The cortices were cut into small pieces using scalpels and were digested for one hour at 37°C on a 55rpm shaker using a pre-warmed papain solution made of 34U/ml of papain and 3.7mg/ml DNase in HALF. The reaction was stopped with 1ml of DMEM plus 10% fetal bovine serum

(FBS). The solution was centrifuged at 200g for five minutes and re-suspended in 1ml of mixed glia culture medium made of 10% FBS and 1/500 Mycozap in DMEM.

The mixed glia was cultured in T75 flasks pre-coated with Poly-D-Lysine-hydrobromide (PDL) in mixed glia culture medium for 11 days. PDL coating of all culture material was carried out by adding 5ug/ml of PDL dissolved in autoclaved double distilled water (ddH_2O) for one hour at 37°C, followed by two washes using ddH_2O in order to remove excess PDL.

During the 11 days the media was changed every three days for six days and consequently every two days. After this time the OPCs were separated from the rest of the cells. Loosely attached microglia were removed by shaking the flasks at 37°C on an orbital shaker at 195rpm for an hour, after which the medium was replaced with new mixed glia medium previously warmed and CO_2 equilibrated. The flasks were left to shake on the same settings for 16h-18h. The media was then transferred to a 10cm non-treated plastic petri dish and placed in the incubator for 15 minutes; this allows excess microglia to attach to the plastic whilst the OPCs remain floating. The supernatant was centrifuged at 300g for five minutes to obtain the OPCs. The supernatant was removed and replaced with serum-free OPC medium, for which the formulation is shown in Table 2.2. The OPCs were then plated according to the experiment that would be carried out. A summary of OPC isolation via mixed glia culture is represented in Figure 2.1.

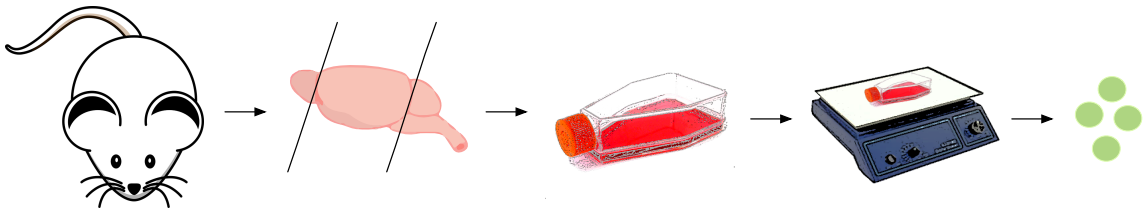


Fig. 2.1 Mixed glia isolation. Rat pup brains are dissected and cultured as mixed glia for 11 days. To detach and isolate OPCs a 'shake-off' is carried out.

Component	**μM**	**MW**
Amino acids		
Glycine	400	75.07
L-Alanine	22	89.09
L-Arginine hydrochloride	483	174.2
L-Asparagine-H2O	5.5	150.13
L-Cysteine hydrochloride-H2O	7.7	313.2
L-Histidine hydrochloride-H2O	200	209.6
L-Isoleucine	802	131.2
L-Leucine	802	131.2
L-Lysine hydrochloride	798	146.2
L-Methionine	201	149.2
L-Phenylalanine	400	165.2
L-Proline	67	115.13
L-Serine	400	105
L-Threonine	798	119
L-Tryptophan	78	204.2
L-Tyrosine disodium salt dihydrate	398	181.2
L-Valine	803	117.2
Vitamins		
Choline chloride	28	139.62
D-Calcium pantothenate	8	238.27
Niacinamide	30	122
Pyridoxine hydrochloride	20	206
Thiamine hydrochloride	10	337
i-Inositol	40	180.2
Inorganic salts		
Ferric Nitrate (Fe(NO3)3”9H2O)	0.25	404
Potassium Chloride (KCl)	5360	74.55
Sodium Bicarbonate (NaHCO3)	880	84
Sodium Chloride (NaCl)	89000	58
Sodium Phosphate dibasic (Na2HPO4) anhydrous	906	120
Zinc sulfate (ZnSO4-7H2O)	0.67	287.56
Other components		
D-Glucose (Dextrose)	25000	180.2
Sodium Pyruvate	227	110.04
MOPS	s10000	269.3

Table 2.1 Formulation for HALF comprising the list of components, their molecular weight and the required μM in solution. HALF was made up in MilliQ water placed on a stirrer. Each component was weighed and added to the solution, making sure that the previous component was dissolved before adding the following one. The pH was adjusted to 7.3 and the solution was filtered through a 0.22μm to make it sterile. HALF was stored at 4°C.

Component	Stock concentration	Final concentration
DMEM/F12	-	1X
N-Acetyl-L-Cystein (NAC)	6mg/ml	60μg/ml
D-glucose	0.56M	25mM
Insulin	4mg/ml	10μh/ml
Sodium pyruvate	100mM	1.5mM
Apo-transferrin	5mg/ml	50μg/ml
Putrescine	1.61mg/ml	16.1μg/ml
Na-Selenite	0.5μg/ml	40ng/ml
Progesterone	6μg/ml	60ng/ml
P/S	6μg/ml	60ng/ml

Table 2.2 Formulation for OPC media. Once all the components were added the solution was filtered through at 0.22μm to make it sterile. Insulin was added after filtering. The media was stored at 4°C.

2.2.3 OPC isolation via magnetic cell sorting

Postnatal day 2-7 SD rats were euthanised via an overdose of intraperitoneal Pentoject injection following the Schedule 1 procedures of the Animal Scientific Procedures Act of 1986. The brains were removed from the skulls and transferred to a petri dish of ice-cod HALF. The cortices were cut into small pieces using scalpels and digested for 30 minutes at 37°C on a 55rpm shaker using a pre-warmed papain solution as for the mixed glia isolation in Section 2.1. The solution was then topped with Hank's balanced salt solution (HBSS)-/- (without calcium and magnesium) and centrifuged at 200g for five minutes.

The supernatant was removed and replaced with a trituration solution made of 2% B27 in HALF. The solution was mixed by inversion and left to settle. This reduced the shear stress on the cells. The first trituration was carried out with a 5ml serological pipette. The triturated solution was left to sit in order for whole tissue pieces to settle. Only the supernatant was transferred to a clean tube via a 70μm strainer. This prevented the transfer of whole tissue parts resulting in a single cell suspension. Another trituration solution made of 0.1% pluronic acid in HALF was used to top up the solution and the procedure was repeated twice more using progressively smaller fire polished Pasteur pipettes.

To remove any myelin debris that would prevent OPC differentiation in the later culture a percoll gradient was used. A 90% percoll solution made using percoll and 10X PBS was added to the single cell solution for a final percoll concentration of 22%. The solution was mixed thoroughly by inversion and centrifuged at 800g for 20 minutes, after which the supernatant was aspirated and the resulting pellet was washed with HBSS-/-. The total number of cells was counted.

Component	Stock concentration	Final concentration
PBS	10X	1X
BSA	7.5%	0.5%
EDTA	0.5M	2mM
Sodium pyruvate	100mM	2mM

Table 2.3 Formulation for MWB used in the MACS isolation. MWB was prepared in autoclaved ddH_2O and the pH adjusted to 7.3. The solution was made sterile by filtering it through a 0.22μm filter. MWB was stored at 4°C.

For every 10^7 cells obtained, the pellet was resuspended in 500μl of Miltenyi Wash Buffer (MBW), 10μg/ml of insulin and 2μg of mouse anti-A2B5 antibody, an established OPC marker. The formulation of MWB is shown in Table 2.3. The solution was incubated for 20 minutes at 4°C and the tube was flicked every five minutes to maintain the cells in suspension. The cells were then washed with HBSS-/-, the supernatant aspirated and the pellet resuspended in 80μl MWB, 10ug/ml of insulin and 20μl of anti-mouse-IgM magAB magnetic beads. This time the solution was incubated for 15 minutes at 4°C and the tube was flicked every five minutes. The cells were then washed with HBSS-/- and resuspended in 1.5ml of MWB and 10μg/ml of insulin regardless of the total cell number after the percoll gradient.

MWB was used to wet the magnetic cell sorting (MACS) column previously placed in a magnetic field. The cell solution was transferred into the column and allowed to drip through. The magnet retains the A2B5$^+$ cells, whilst all the other cells dripped through. When the elution stopped, the column was washed with clean MWB plus 10μg/ml of insulin to get rid of any excess non-specific cells still trapped in the column. Once the elution completely stopped, the column was removed from the magnet and placed in a clean tube, pre-warmed OPC medium was added to the column and the OPCs were eluted using a plunger. The OPCs were plated on PDL-coated material according to the experiment that was being carried out. A summary of OPC isolation via MACS is represented in Figure 2.2.

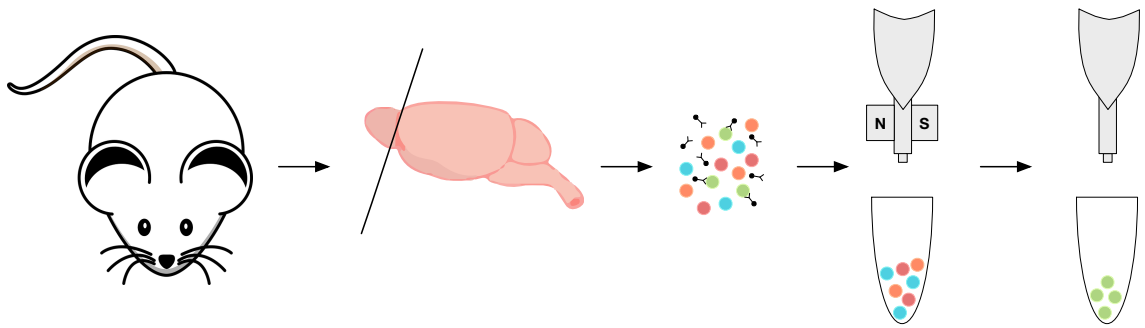


Fig. 2.2 MACS isolation. Rat pup brains are dissected and a single cell suspension is obtained. The OPCs are isolated using antibodies against A2B5, an OPC marker, followed by an incubation with secondary antibodies conjugated to magnetic beads. By running the cell suspension through a column in a magnetic field the non-specific cells are eluted and the OPCs are retained. These can be eluted separately to obtain a pure fraction of OPCs.

2.2.4 FBS charcoal treatment

To prepare the charcoal, activate charcoal was diluted 2.5% w/v in sterile 1X PBS and kept in agitation at 4°C for at least 12 hours. The solution was centrifuged five minutes at 500g, the supernatant removed and the wet charcoal was washed twice with sterile H_2O. The wet charcoal was incubated with FBS for a final concentration of 2.5% w/v and kept in agitation at 4°C for 18 hours. The solution was then passed through a 0.4μm strainer until all the charcoal was removed and FBS depleted in retinoid and steroid molecules was obtained. This FBS was used to prepare charcoal-treated mixed glia media.

2.2.5 Small molecule treatment

After isolation, OPCs were cultured in serum-free OPC medium in a 37°C incubator at 5% CO_2. They were left to recover overnight from the stress of the MACS with 10ng/ml PDGF-AA and 10ng/ml bFGF. The following day the OPC medium was changes and nuclear receptor agonists or antagonists were added to the culture. The final concentration and the duration of the treatment are specified for individual experiments reported in Chapter 3. All treatment stocks were prepared in DMSO at a concentration such that the final DMSO concentration in culture would be 0.1%. To assess proliferation OPCs were treated for four days and to assess differentiation they were treated for six days *in vitro*.

2.2.6 EdU incubation

Ethynyl-2'deoxyuridine (EdU) was diluted in media to a 20μM concentration and left to warm and equilibrate at 37°C in the incubator. Once equilibrated, half of the medium in the well was replaced with an equivalent volume of fresh medium plus EdU, for a final EdU concentration of 10μM. A complete change of media can cause cells to alter their cell cycle; by changing only half of it this chance is reduced. The live cells were incubated with EdU for four hours before fixation as reported in Section 2.2.8.

2.2.7 Isolation purity assessment using flow cytometry

OPCs were isolated using MACS. After elution the cells were divided equally between the samples to stain and the controls. Approximately 300,000 cells per tube were obtained. The cells were stained for cell death, OPCs, oligodendrocytes, microglia and red blood cells. For each antibody used there was the equivalent fluorescence minus one (FMO) control. The live cells were firstly stained in the dark for 15 minutes at room temperature (RT) with zombie violet (ZV) diluted 1/100 in 1X PBS. The cells were topped up with 1X PBS and spun at 350g for five minutes. The cells were then incubated in 100μl of 1/100 relevant primary antibodies for 20 minutes at 4°C. The tubes were topped up with 0.5% BSA in 1X PBS and spun down at 300g for five minutes. The cells were incubated in the appropriate secondary antibodies for 15 minutes at RT. The cells were spun down at 300g for five minutes, resuspended in 500μl of 0.5% BSA in 1X PBS and sorted on a BD Influx cell sorter, equipped with 488nm, 640nm, 561nm and 405nm lasers. Results were analysed using FlowJo, LLC, a software for single-cell flow cytometry analysis.

The staining protocol was set up by Natalia Murphy, the staining was carried out by Alerie Guzmán de la Fuente (both members of the Franklin laboratory) and the cells were flowed by the CIMR flow facility.

Antibody	Dilution	Supplier	Cat. number
Primary antibodies			
PE conjugated mouse anti-A2B5 (IgM)	1:11	Miltenyi Biotec	130-093-581
PECy5.5 conjugated mouse anti-CD11b	1:100	BioLegend	201820
Goat anti-MOG	1:40	R&D systems	BAF2439
Mouse anti-OX83	1:50	BioLegend	250402
Secondary antibodies			
PE/Cy7 rat anti-mouse	1:500	BioLegend	406614
A647 donkey anti-goat	1:800	Invitrogen	A21447

Table 2.4 Antibodies used to assess purity of the MACS isolation using flow cytometry.

2.2.8 Immunocytochemistry

Cells were fixed with 4% paraformaldehyde (PFA) at room temperature (RT) for 10 minutes and washed twice with 1X PBS. They were then blocked in blocking solution made up of 5% normal donkey serum and 0.1% Triton-X 100 in 1X PBS for one hour at RT. If the cells had been previously exposed to EdU, EdU detection was carried out by incubating the coverslips for 30 minutes with the Click-iT reaction cocktail (Reaction Buffer, CuSO_2, Alexa Fluor azide 488 and Reaction Buffer Additive) at RT as described in the Click-iT EdU Alexa Fluor Imaging Kit. The coverslips were washed once with 1X PBS. The cells were then incubated with the relevant primary antibodies diluted in blocking solution for 16 hours at 4°C. Excess primary antibody was washed away with 1X PBS and the cells were incubated with secondary antibodies diluted in 1X PBS for one hour at room temperature. Excess secondary antibody was washed away with 1X PBS. The cells were then incubated for 10 minutes at RT with 2μg/ml of Höechst in 1X PBS. The cells were washed twice with 1X PBS and once with ddH_2O. If the cells were on coverslips they were then mounted on polysine slides with Fluoromount G. The slides were left to dry overnight at 37°C. Immunocytochemistry pictures were taken using the Leica SP5 Confocal microscope at 10X, 20X or 40X magnification, or using the GE Healthcare IN Cell Analyzer 2200 at either 10X and 20X magnification. Quantification of the images generated was carried out using CellProfiler, cell imaging analysis software. A list of the antibodies used for immunocytochemistry is shown in Table 2.5.

2.2.9 Co-immuno precipitation

Co-immunoprecipitation (CoIP) was carried out using the Pierce Crosslink Immunoprecipitation Kit using 500μg of total protein per sample. As a pre-clearing step, 20μl of cross linked 4% beaded agarose were added to the 500μg protein sample. The sample was left to shake at maximum speed at 4°C for an hour. This step aids in clearing any non-specific binding of the sample that could bind to the resin beads. To bind the primary antibody to the resin beads 20μl of A/G plus agarose beads were added to the column and centrifuged at 2000g for 1 minute at 4°C. The column was washed twice with 1X Coupling Buffer after which the bottom of the column was plugged and 10μg of desired primary antibody were added to the column. The antibody was topped up using 1X Coupling Buffer to a final volume of 100μl. The column was plugged at the top and incubated on a rotator for 1 hour at room temperature. After five washes with 1X Coupling Buffer the bound antibody was crosslinked by incubating the column with 9μl of 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) diluted in DMSO. DSS was further diluted to a final concentration of 2.5mM by

Antibody	Dilution	Supplier	Cat. number
Primary antibodies			
Rabbit-anti Olig2	1:1000	Millipore	AB9610
Goat-anti Olig2	1:200	R&D Systems	AF2418
Rat-anti MBP	1:500	Serotec	MCA4095
Rabbit-anti NG2	1:200	Millipore	MAB5320
Rabbit-anti ENPP6	1:100	Abcam	ab87587
Mouse-anti O4 (IgM)	1:500	R&D Systems	MAB1326
Mouse-anti CNPase	1:500	Sigma Aldrich	c5922
Rat-anti GFAP	1:1000	DAKO	Z0334
Mouse-anti CD11b	1:300	Serotec	MCA275R
Rabbit-anti p27	1:800	Cell Signalling	3686
Secondary antibodies			
Alexa 488 donkey anti-rat	1:500	Invitrogen	A21208
Alexa 647 donkey anti-rabbit	1:500	Invitrogen	A31573
Alexa 488 donkey anti-rabbit	1:500	Invitrogen	A21206
Alexa 568 donkey anti-rabbit	1:500	Invitrogen	A10042
Alexa 568 donkey anti-goat	1:500	Invitrogen	A11057
Alexa 488 donkey anti-mouse	1:500	Invitrogen	A21202
Alexa 568 donkey anti-mouse	1:500	Invitrogen	A10037
Alexa 647 donkey anti-mouse	1:500	Invitrogen	A31571
Alexa 488 goat anti-mouse IgM	1:500	Invitrogen	A21042

Table 2.5 Antibodies used for *in vitro* experiments. All antibodies are IgG subclass unless specified otherwise.

adding 41μl of 1X Coupling Buffer directly to the column, which was once again plugged and incubated on a rotator for 1 hour at room temperature. The column was washed four times with Elution Buffer followed by another four washes with Lysis/Wash Buffer and the bottom was plugged once again. The protein sample was spun at 2000g for 1 minute at 4°C, and the supernatant was added to the column. The top was plugged and the column was incubated on a rotating platform at 4°C for 16 hours, after which the column was washed three times with Wash/Lysis Buffer and once with 1X Condition Buffer. The column was placed in clean eppendorf tubes and 10μl of Elution Buffer were added to the column and spun through. Another 15μl of Elution Buffer were added to the column, which was left to incubate for 5 minutes at room temperature. The column was centrifuged, resulting in 25μl of flow through containing the proteins bound to the protein of interest.

2.2.10 Mass spectrometry

OPCs isolated via MACS were washed once with 1X PBS and lysed with a lysis solution made with Lysis Buffer and 1X HALT protease inhibitor. CoIP was carried out for RXRγ using rabbit anti-RXRγ from Abcam (ab15518) primary antibody. The solute was then taken to GSK for further processing and mass spectrometry analysis which was carried out by Carla Newman. For the in-solution digest, samples were reduced, alkylated and digested as described by Lopez- Ferrer et al., 2006. Samples were then desalted using StageTips. Samples were run on a QExactive via EasySpray nanoelectrospray ion source, coupled to an easy-nLC 1000 using a Acclaim PepMap 100 75µm x 2cm nanoViper pre-column and an Easy PepMap 75µm x 15cm C18, 2µm, 100A chromatography column. For both, the mobile phases were 0.1% formic acid in waterford the aqueous phase and acetonitrile for the organic phase. The gradient time was 1 hour, the column temperature 40°C and data was acquired using a Top 15 method, where HCD fragmentation was used to sequence the top 15 ions per full scan spectrum. Additionally, the instrument was set to 2% underfill ratio and 3m/z isolation window. Results were further processed using Scaffold Proteome 2.2 Software to analyse the raw data and Proteome Discoverer 1.4 to search through databases.

2.2.11 Proximity ligation assay using Duolink®

Cells isolated via mixed glia were fixed with 4% PFA for 10 minutes and washed twice with 1X PBS. The cells were then blocked in Duolink® Blocking Reagent for 30 minutes at 37°C. The cells were consequently incubated with antibodies against nuclear receptors or co-regulators diluted in Duolink® Antibody Diluent for 16 hours at 4°C. The cells were washed for five minutes in Buffer A twice and incubated with anti-mouse PLA Plus and

Antibody	Dilution	Supplier	Cat. number
Primary antibodies for NRs			
Rabbit-anti RXRγ	1:100	Abcam	ab15518
Mouse-anti RXRγ	1:100	Santa Cruz	sc-514134
Rabbit-anti VDR	1:100	Santa Cruz	sc-1008
Rabbit-anti RARβ	1:100	Santa Cruz	sc-552
Rabbit-anti RARγ	1:100	Santa Cruz	sc-7389
Rabbit-anti THRα	1:100	Abcam	ab53729
Rabbit-anti THRβ	1:100	Abcam	ab5622
Mouse-anti PPARβ	1:100	Santa Cruz	sc-74440
Rabbit-anti PPARγ	1:100	Santa Cruz	sc-7196
Rabbit-anti LXRα	1:100	Abcam	ab3585
Mouse-anti LXRβ	1:100	Abcam	ab76983
Rabbit-anti Nurr1	1:100	Santa Cruz	sc-990
Primary antibodies for co-regulators			
Mouse-anti p300	1:100	Novus Biologicals	RW105
Mouse-anti PGC1α	1:100	Millipore	ST1202
Mouse-anti Brg1	1:100	Santa Cruz	sc-374197
Mouse-anti TRAP220	1:100	Santa Cruz	sc-74475
Mouse-anti HDAC1	1:100	Cell Signaling	5356
Mouse-anti HDAC2	1:100	Cell Signaling	5113S
Mouse-anti HDAC3	1:100	Cell Signaling	3949
Mouse-anti HDAC11	1:100	Santa Cruz	sc-390737
Mouse-anti NCoR	1:100	Millipore	MABE570
Mouse-anti SIRT1	1:100	Cell Signaling	8469
Mouse-anti SIRT2	1:100	Santa Cruz	sc-28298
Mouse-anti SRC1	1:100	Santa Cruz	sc-73057

Table 2.6 Antibodies used for *in vitro* proximity ligation assay using Duolink®. All antibodies are IgG subclass unless specified otherwise.

anti-rabbit PLA Minus probes diluted 1/5 in Antibody Diluent for one hour at 37°C. After another two five minute washes of Buffer A the cells were incubated with Duolink® Ligation solution made of Ligation Buffer diluted 1/5 in DNase-free water and ligase diluted 1/40 for 30 minutes at 37°C. The cells were washed twice with Buffer A and were incubated with Amplification Stock diluted 1/5 in DNase-free water and polymerase diluted 1/80 for 1:40 hours at 37°C. The cells were then washed for 10 minutes with 1X Buffer B twice and once with 0.01X Buffer B for one minute. The cells were incubated for EdU detection and with OPC/OLG markers as described in Section 2.2.8. A list of the antibodies used for Duolink® is shown in Table 2.6.

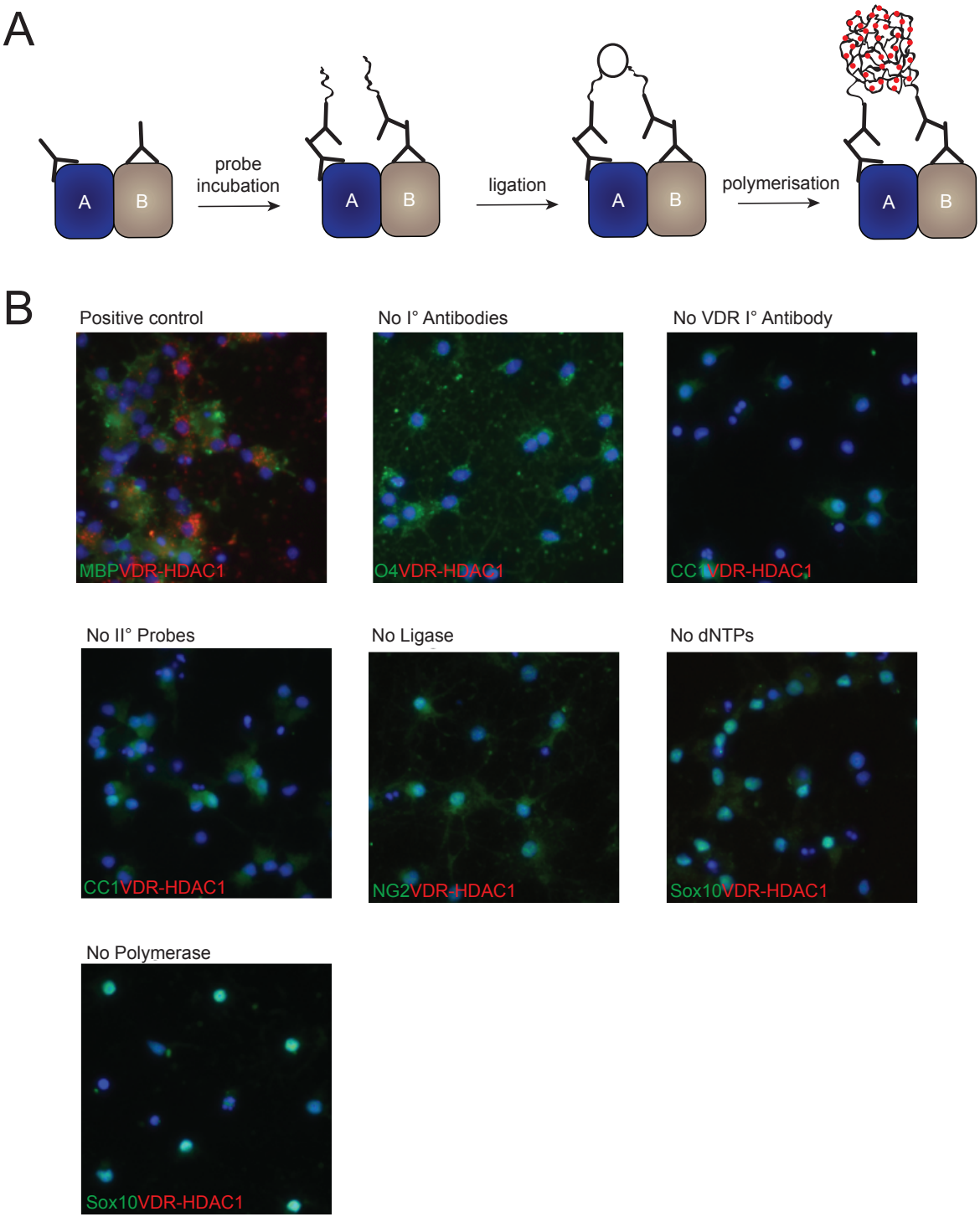


Fig. 2.3 Duolink® *in situ* proximity ligation assay technology. (A) The blue and grey shapes represent two hypothetical proteins of interest named A and B. These are bound with relevant primary antibodies and with secondary probes with DNA sequences associated to them. If the proteins are close enough to be considered interacting, the probes will ligate in the presence of a ligase enzyme. The distance between the two epitopes can be up to 40nm for ligation to happen. The signal is amplified using a polymerase enzyme and fluorescent dNTPs represented here in red. The signal will be fluorescent and can be imaged with a fluorescent microscope. (B) Panel of technical controls for Duolink® with a variety of oligodendrocyte lineage markers.

2.2.12 Cell cycle analysis using flow cytometry

OPCs were isolated using MACS, plated in T75 flasks and left to recover overnight. The next morning they were treated with either 9cRA, T3 or DMSO for 48 hours in the presence of growth factors (GF). The cells were then dissociated by removing the media and incubating the cells with 1ml of 1X TrypLE for 6 minutes at 37°C. The cells were pipetted up and down to fully detach them from the flask, transferred to a falcon tube and were spun at 300g for 5 minutes. The cells were then transferred to low binding microcentrifuge tubes. The cells were stained with a live/dead stain by incubating them with ZV 1/100 in 1X PBS for 15 minutes at room temperature in the dark. They were washed once with 1X PBS and then fixed using 70% EtOH in 1X PBS for 2 hours at 4°C. The OPCs were then washed with 1X PBS and were incubated with a propidium iodide (PI) solution (PI 1/200 + RNase 1/100 in 1X PBS) for 30 minutes at 37°C. The cells were then transferred to flow tubes and cell cycle stages were assessed using a BD Fortessa instrument equipped with 405nm and 561nm lasers. Results were analysed using the 'cell cycle analysis' feature of FlowJo, a software for single-cell flow cytometry analysis.

2.2.13 OPC dissociation

The media was removed and the OPCs were washed once with pre-warmed 1X PBS to remove any remaining media. A pre-warmed dissociation solution made of 1/100 of papain and 40μg/ml of DNase in HALF was added to the OPCs which were placed back in the incubator for three minutes. The flasks were banged enough for the cells to detach. The papain reaction was stopped by adding the the same amount of DMEM plus 10% FBS. The cells were transferred to a falcon tube and spun at 300g for five minutes, counted and were then used for ChIP sequencing.

2.2.14 ChIP sequencing

OPCs were isolated using MACS and were left to proliferate in T75 flasks in GF presence. After five days the OPCs were treated for 24 hours with either 50nM 9cRA or 1/1000 DMSO, dissociated according to Section 2.2.13 and ChIP was carried out following the method described. For each sample five million OPCs were used. The OPCs were fixed in 1% PFA for 10 minutes and the reaction was stopped by adding glycine for 5 minutes at RT. The cells were washed once with 1X PBS and lysed using a solution of 100μl SDS Lysis Buffer and 0.5μl Protease Inhibitor Cocktail II per 1 million cells. From then on all samples were kept on wet ice to prevent dissociation of transcription factors from the chromatin. The chromatin

was then sheared using the Bioruptur*Plus* sonicator: OPCs were at a concentration of 1 million OPCs in 100μl and were sheared on the high power setting for six minutes composed of 30 seconds off, 30 seconds on cycles at 4°C. This resulted in chromatin pieces between 400bp-200bp. Samples were spun at 13000g for 10 minutes at 4°C and transferred to clean microfuge tubes to remove any insoluble material. The samples were then pre-cleared of any unspecific stickiness by incubating them with Protein G Agarose for an hour at 4°C with rotation. The samples were spun at 4000g for one minute to pellet the agarose and the supernatant was transferred to a new microfuge tube. The samples were incubated overnight on a rotator at 4°C with 10μg of either a mouse anti-RXRγ antibody or a mouse normal IgG antibody as the negative control. Protein G Agarose was added to the samples and incubated for an hour at 4°C with rotation after which the agarose was pelleted by spinning the samples at 4000g for one minute and the supernatant was discarded. The agarose beads were then washed with five minute washes using a series of buffers in the following order: one wash of Low Salt Immune Complex Wash Buffer, one wash of High Salt Immune Complex Wash Buffer, one wash of LiCl Immune Complex Wash Buffer and two washes of TE Buffer. The protein-DNA complexes were eluted by incubating the agarose beads with 100μl of elution buffer (10μl of 20% SDS, 20μl of 1M $NaHCO_3$, 170μl of sterile H_2O) at RT for 15 minutes. The agarose was pelleted and the supernatant was transferred to a clean tube. This step was carried out twice. The protein-DNA crosslinks were then reversed by incubating the samples overnight at 65°C with 8μl of 5M NaCl and all RNA and protein was degraded by firstly incubating the samples with 1μl RNase A for 30 minutes at 37°C and consequently incubating with 4μl EDTA, 8μl 1M Tris-HCl and 1μl Proteinase K for two hours at 45°C. The remaining DNA was then purified by adding Binding Reagent A to the samples and spinning the solution through a filter column at 13000g for 30 seconds at RT. The column was washed with 500μl of Wash Reagent B and finally the DNA was eluted by adding 50μl of Elution Buffer C and spinning the column at 13000g for 30 seconds at RT. The DNA was further purified and concentrated for sequencing as described in Section 2.2.15.

Quantification of the material was carried out using a Quibit and the samples were paired end sequenced with a read length of 150 on one lane of a HiSeq 4000. This was carried out by the NGS library facility at the Cambridge Stem Cell Institute.

The subsequent peak calling was carried out by Sabine Dietmann. Paired-end read files were quality-trimmed using *TrimGalore* (options: `-q 30 -length 15`) discarding reads shorter than 15 nucleotides. Reads were aligned with Bowtie2 (option: `--sensitive -x`) against the rat reference genome (rn6). Potential PCR duplicates were removed using Picard tools 'MarkDuplicates' (https://broadinstitute.github.io/picard). UCSC genome browser profiles were generated using bedtools "genomeCoverageBed".

Peaks were called with model basis analysis of ChIP-seq 2 (options: `-q 0.5`). Peaks called for three replicates were intersected and ranked by fold enrichment. Only peaks that were called in two or more replicates were considered for further analysis.

2.2.15 Concentrating DNA

To concentrate DNA the in solution purification protocol of the 'QUIAEX II Gel Extraction Kit' was followed. Three volumes of Buffer QX1 was added to on volume of sample. After adding 15μl of QIAEX II, the sample was incubated at RT for 10 minutes and mixed every two minutes. The sample was centrifuged at maximum speed for 30 seconds and the supernatant removed. The pellet was washed twice with 50015μl of Buffer PE, the supernatant was removed and the pellet air-dried for 15 minutes. After this, 20μl of RNase and DNase-free H_2O was added, the pellet vortexed and incubated at RT for five minutes. The sample was centrifuged and the supernatant with the purified and concentrated DNA was placed into a new tube.

2.2.16 Western blot

Cell lysates were obtained by removing OPC media, washing the cells once with 1X PBS and adding the lysis solution made of Lysis Buffer and 1X HALT protease inhibitor. The cells were gently scraped using a cell scraper and the solution was collected and placed on wet ice for 10 minutes. The protein concentration was measured using the BCA assay; for this the protocol of the 'BCA Assay Kit'. Standards were prepared using the Lysis Buffer as diluent and BSA. After preparing the standards and samples, the BCA working reagent was prepared according to the protocol and added to the samples which were placed at 37°C for 30 minutes. The protein concentration was then measured using the Nanodrop set at an absorbance of 562nm. The samples and mixed with LDS loading buffer and 1X Reducing Agent and boiled for 10 minutes at 95°C after which the desired protein amount (5-15μl) was loaded on 4-12% Bis-Tris gels alongside the Precision Plus standard protein ladder. The gels were run at 100V for one hour and 20 minutes in 1X MOPS Running Buffer made up in dH_2O. When the gel finished running it was removed from the tank and transferred onto a polyvinylidene difluoride (PVDF) membrane previously activated for one minute in 100% MeOH. The two were placed between two pieces of blotting paper previously soaked in freezing cold Transfer Buffer (150ml of 10X Transfer Buffer, 300ml EtOH and up to 1.5L of dH_2O) and all was placed in a holder. The holder was then placed in a transfer tank which was filled with Transfer Buffer. The transfer was run at 100V for 90 minutes. The membranes were removed from the holder and blocked in blocking solution made of Odyssey blocking

Antibody	Dilution	Supplier	Cat. number
Primary antibodies			
Rabbit-anti RXRγ	1:100	Abcam	ab15518
Rabbit-anti RXRγ	1:100	Abcam	ab15519
Rabbit-anti RXRγ	1:100	Santa Cruz	sc-365252
Rabbit-anti RXRγ	1:100	Santa Cruz	sc-555
Mouse-anti RXRγ	1:100	Santa Cruz	sc-514134X
Rabbit-anti VDR	1:100	Santa Cruz	sc-1008
Rabbit-anti LXRα	1:100	Abcam	ab3585
Rabbit-anti Nurr1	1:100	Santa Cruz	sc-990
Rabbit-anti THRα	1:100	Abcam	ab53729
Rabbit-anti THRβ	1:100	Abcam	ab5622
Rabbit-anti NG2	1:100	Millipore	AB5320
Rabbit anti-p27	1:100	Cell Signaling	3686
Rabbit anti-cyclin D1	1:500	Cell Signaling	2978
Rabbit anti-cyclin E1	1:500	Cell Signaling	20808
Mouse-nIgG	1:100	Millipore	12-371B
Rabbit-nIgG	1:100	Cell Signaling	2729
Mouse peroxidase-anti actin	1:20,000	Sigma Aldrich	A3854
Secondary antibodies			
IRDye 680RD donkey anti-rabbit	1:10,000	Li Cor	925-68073
IRDye 800CW donkey anti-mouse	1:10,000	Li Cor	926-32212

Table 2.7 Antibodies used for western blot experiments. All antibodies are IgG subclass unless specified otherwise.

buffer and TBS-T (1:1) for one hour at RT on a shaker. The membranes were then incubated with the relevant primary antibody in blocking solution for 16 hours at 4°C, after which the membranes were washed with TBS-T and incubated in the dark with the relevant secondary antibodies. The membranes were washed again and imaged on the Li-Cor Odyssey Blot Imager with a two minute exposure. They were were then incubated with anti-actin primary antibody conjugated to peroxidase for 20 minutes at RT and washed twice. The membrane was then incubated with a solution of Detection Reagents 1 and 2 (mixed 1:1) from the ECL Western Blotting Analysis System. The membranes were imaged on Li-Cor Odyssey Blot Imager with a two minute exposure on the chemiluminescence setting. Intensity of the bands was measured using the 'measuring tool' on the Odyssey software. A list of the antibodies used for western blot is shown in Table 2.7.

2.2.17 RNA extraction and purification

Cells isolated via MACS were cultured in wells of a six-well plate and treated with the relevant small molecules for 24 hours. The cells were washed once with 1X PBS and lysed using 300μl of Trizol per well at 4°C on a shaker for 10 minutes. The solution was collected and frozen at -80°C until RNA purification.

The samples were left to defrost on wet ice and the RNA was purified using 'Direct-zol RNA MicroPrep'. All centrifugations were carried out at 13500g for 30 seconds. 300μl of 99% molecularly pure ethanol was added to each tube and the sample was mixed thoroughly. The mixture was transferred to a spin column inside a collection tube and centrifuged. The column was placed in a fresh collection tube and was washed twice with 400μl of RNA Pre-Wash solution. The flow through was discarded and 700μl of RNA Wash Buffer were added to the column and the column was centrifuged for 2 minutes, after which the column was transferred to an RNase-free tube. To elute the RNA 15μl of DNase/RNase-free H_2O were directly added to the column and centrifuged. The amount of RNA obtained was measured using a NanoDrop instrument and was stored at -80°C or immediately converted to cDNA.

2.2.18 cDNA synthesis

To isolate cDNA from RNA the 'QuantiTect Reverse Transcription Kit' was used. The isolated RNA template and kit components were left to defrost on wet ice. First the genomic DNA was eliminated from the samples using a solution of 2μl of 7X gDNA Wipeout Buffer and up to 1μg of RNA template. The solution was mixed on wet ice and made up to to 14μl with RNase-free H_2O and incubated for 2 minutes at 42°C. The mixture was then placed on wet ice. A second mixture was made using 1μl of reverse transcriptase, 4μl of 5X reverse transcriptase buffer, 1μl of reverse transcriptase primer mix and the whole 14μl of the first mix. This was also prepared on wet ice. After mixing, the solution was incubated at 42°C for 15 minutes and for another 3 minutes at 95°C. The samples were used for qPCR.

2.2.19 Quantitative PCR

SYBR Green qPCR protocol was used for carrying out qPCR. For each well of a 384 well-plate the set up shown in Table 2.8 was used, and either 1μg or 3μg of cDNA were used. Samples were pipetted on wet ice and triplicates were prepared for each sample. Upon completion of the sample preparation the plate was sealed, vortexed briefly and centrifuged in order to collect the liquid at the bottom of the well. The qPCR was run on a QuantStudio 7 Flex machine, ThermoFisher, using the ΔΔCT program with the parameters shown on Table 2.9. Results were analysed using QuantStudio 6 and 7 Flex Software, ThermoFisher. If the primer efficiency was between 90-110%, ΔΔCT approximation was used for the analysis.

Component	**Volume**
cDNA template	2μl
2X SYBR Green Mix	10μl
2μM forward primer	2μl
2μM reverse primer	2μl
H_2O	4μl

Table 2.8 Set up for qPCR run.

Step	**Temperature**	**Time**
Initial denaturation	94°C	2 minutes
40 cycles as follows:		
Denaturation	94°C	15 seconds
Annealing, extension and read fluorescence	60°C	1 minute
Hold	4°C	Infinite

Table 2.9 Parameters for the qPCR run.

Target	Primer code	Primer type	Sequence 5' to 3'
CDK1	FR1 Cdk1	Forward	CGCTCGTTAAGAGTTACTTG
	RR1 Cdk1	Reverse	CTCTTATCGGTATTCCAAACG
CDK18	FR1 Cdk18	Forward	AAACATACGTGAAACTGGAC
	RR1 Cdk18	Reverse	CAGAGACACCTCTCGAATAG
CDKN1b	FR1 Cdkn1b	Forward	AAAATTTGAATAATCGCCACAG
	RR1 Cdkn1b	Reverse	GAGTTTTGCCCAGTGTTATC
MKI67	FR1 Mki67	Forward	AAAAGACAAAGAAGACCCAG
	RR1 Mki67	Reverse	AGTCCATTTTCCAGTTTAGC
WNT7a	FR1 Wnt7a	Forward	ATCATCGTCATAGGAGAAGG
	RR1 Wnt7a	Reverse	ATAATTGCATAGGTGAAGGC
PDGFRL	FR1 Pdgfrl	Forward	ACCTACATCTTCTTCACAGAG
	RR1 Pdgfrl	Reverse	GTTCAGGTAGACAACATCAAAG
BMP4	FR1 Bmp4	Forward	AAAAATTATCAGGAGATGGTGG
	RR1 Bmp4	Reverse	AAAGTCCAGCTATAGGGAAG
FGFR4	FR1 Fgfr4	Forward	CTGTGAAGATGCTGAAAGAC
	RR1 Fgfr4	Reverse	GTTCTTGTGTCTTCCGATTAG
ENPP6	FR1 Enpp6	Forward	AATTTGTCTCTCCTTTGACC
	RR1 Enpp6	Reverse	CTTTCTGGACATCAGATAGC
MOBP	FR1 Mobp	Forward	ACAACTTCAGCTTGTTTGAG
	RR1 Mobp	Reverse	TGTGTGTCCTTCTCTCTTTC

Table 2.10 Forward and reverse primer sequences for the qPCR targets selected. All primers were pre-designed and purchased from Sigma Aldrich.

2.2.20 Statistical analysis

All statistical analysis was carried out using GraphPad Prism 7 (GraphPad Software, Inc.). The D'Agostino-Pearson omnibus test and Saphiro-Wilk test were carried out to assess how far the distribution of the results obtained was from a Gaussian distribution. When comparing two groups where the data followed a normal distribution, Unpaired Student t Test was used assuming two-tailed distribution. When comparing more than two groups with a single variable where the data followed a normal distribution, ordinary one-way analysis of variance (ANOVA) was carried out followed by an appropriate post-hoc test where significance was reached for individual groups. In cases where the data did not follow a normal distribution, the Kruskal-Wallis test was used instead. If two or more variables were present, ordinary two-way ANOVA was used to assess interaction of the variables. If these were positive, ordinary one-way ANOVAs were carried out for each variable separately. Results were considered significant if the p value was smaller than 0.05, with significance as follows: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p < 0.0001$. In Chapter 3 the statistical test used, the number of biological replicates (n) and the p value are indicated for each experiment.

Chapter 3

Results

3.1 Mass spectrometry analysis of RXR complexes in OPCs

RXRγ heterodimers are important in OPC biology due to the influence they have on oligodendrocyte lineage progression. They function via the recruitment of additional transcription factors and co-regulators, resulting in the construction of complexes involved in aiding chromatin remodelling and gene transcription. Due to both their high conservation and ubiquitous expression, NRs elicit cell-specific effects in two principal ways: by expressing isoforms of single receptors in a tissue-specific manner and by forming function-specific complexes via the recruitment of the required set of co-regulators (Braissant, 1996; Glauser and Barakat Walter, 1997; Mukherjee et al., 1997; Smith et al., 1997; Mollard et al., 2000; Liu et al., 2002).

Thus, to validate the partner switching hypothesis and assess how RXRγ heterodimers control OPC differentiation, it is necessary to understand:

1. whether RXRγ associates equally with all its partners or if its binding is temporally skewed towards a specific subset of NRs as OPCs differentiate;

2. which components make up the overall complexes formed throughout the various stages of differentiation.

As the partners and regulators will be associated in a cell-specific manner, I decided to answer the above questions by adopting a non-biased approach involving mass spectrometry.

The experimental strategy consisted in acutely isolating primary neonatal rat OPCs via MACS, lysing them and carrying out CoIP for RXRγ, thereby obtaining a solution enriched in RXRγ and the proteins associated to it. The solution would be then taken for in-solution mass spectrometry analysis. This method is highly sensitive and reduces sample wastage

as it does not require the sample to be handled further, unlike with mass spectrometers that conversely require running a gel prior to analysis. Despite the high sensitivity of the mass spectrometer, it is necessary to obtain as many primary cells as possible in order to carry out the CoIP with enough protein lysate, hence the use of neonatal OPCs. In the developing brain, OPC numbers increase postnatally, resulting in the isolation of approximately 2 million OPCs per neonatal brain at P7, a much higher number when compared with OPC isolation from adult rats, typically yielding half this quantity per brain. Furthermore, neonatal OPCs are *bona fide* stem cells and are typically used as reference for the development of remyelination therapies.

3.1.1 Purity and viability of acutely isolated OPCs

To assess the purity of my MACS isolation, flow cytometry analysis was performed on the fraction eluted at the end of the isolation, termed "positive fraction". The OPCs were sorted using the marker A2B5 rather than the more commonly used OPC marker PDGFRα, as the antibodies available typically recognise the internal fraction of the receptor. Additionally, the expression of PDGFRα decreases with ageing. Therefore, A2B5 provides a more consistent OPC yield in ageing studies. NG2, another potential marker, is also expressed by other cell types, including microglia, pericytes and endothelial cells (Pouly et al., 2001; Yokoyama et al., 2006; Guimarães-Camboa et al., 2017). I then determined the viability of the isolated cells using the live/dead stain ZV, and calculated the percentage of OPCs ($A2B5^+$), microglia ($CD11b^+$), red blood cells ($OX83^+$), oligodendrocytes (MOG^+) and OPCs on their way to becoming oligodendrocytes ($A2B5^+$ and MOG^+). The staining protocol was developed by Natalia Murphy and was carried out by Alerie Guzmán de la Fuente, both members of the Franklin laboratory.

In order to gate correctly, cells from an adult rat brain were used; this was to ensure the presence of any other cell type that could contaminate the sample preparation, especially MOG^+ oligodendrocytes. Figure 3.1A illustrates the gating strategy adopted: the control used to set the gates is shown in black, whilst the cyan sample overlaid is a neonate brain sample. The quantification in Figure 3.1B revealed that almost all cells are viable immediately following the elution of the positive fraction, with 99.67% of single cells not presenting ZV stain (mean=99.67%, SD=0.06, n=3).

Further analysis focused on assessing what cell types the live cell population consisted of. The results are shown in Figure 3.1C. A2B5 was the marker used for the isolation of OPCs, and 87.47% of the total live population resulted in being exclusively $A2B5^+$, suggesting a high enrichment in OPCs. With regards to other oligodendrocyte lineage cells, almost no mature or newly forming oligodendrocytes were present in the elution of neonatal brains

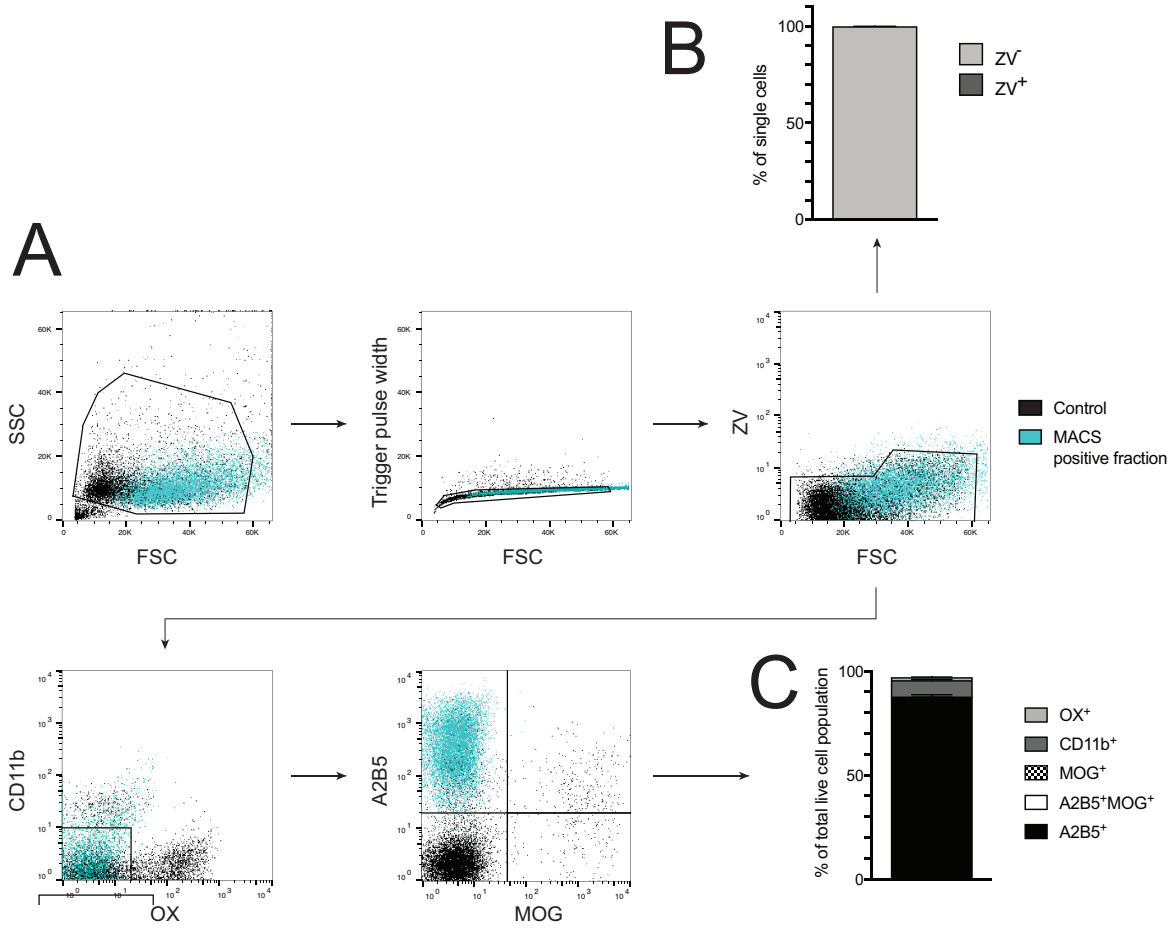


Fig. 3.1 Purity of acutely isolated OPCs. In panel (A) the gating strategy adopted for each marker is shown. Initial gating was done on a control adult brain (shown in black) to ensure the presence of mature oligodendrocytes. The plots are additionally overlaid with one of the neonatal MACS positive fractions obtained (shown in cyan). The side scatter, forward scatter and trigger pulse width were initially used to determine what was a true single cell based on size. Once that was determined, ZV was used to distinguish between live and dead cells. (B) This resulted in an extremely high cell viability out of the total single cell population (mean=99.7%, SD=0.06, n=3). The live population was plotted against CD11b and OX83, resulting in the plot of three distinct populations. The population that was double negative was then plotted against A2B5 and MOG. Neonatal OPCs present a large A2B5 high population and almost no MOG-expressing cells. The composition of the live population is quantified in bar graph (C). It is made up of 8.13% ± 0.71% $CD11b^+$, 1.41% ± 0.17% $OX83^+$, 0.03% ± 0.004% MOG^+, 0.03% ± 0.003% $A2B5^+MOG^+$, 2.39% of cells unaccounted for and 87.47% ± 2.14% $A2B5^+$. All data is presented as mean ± SEM, biological n=3.

(on average 0.03% for both). Nearly no red blood cells were found, with the percentage of $OX83^+$ cells being on average only 1.41%. The microglial fraction made up on average 8.13% of the live cell population, which is a higher percentage than other non-specific cell types pulled down. However, this is still a relatively low percentage compared to the number of $A2B5^+$ cells. This leaves 2.39% of live cells unaccounted for. These could be astrocytes, pericytes or neurons: however, this remains a very small percentage.

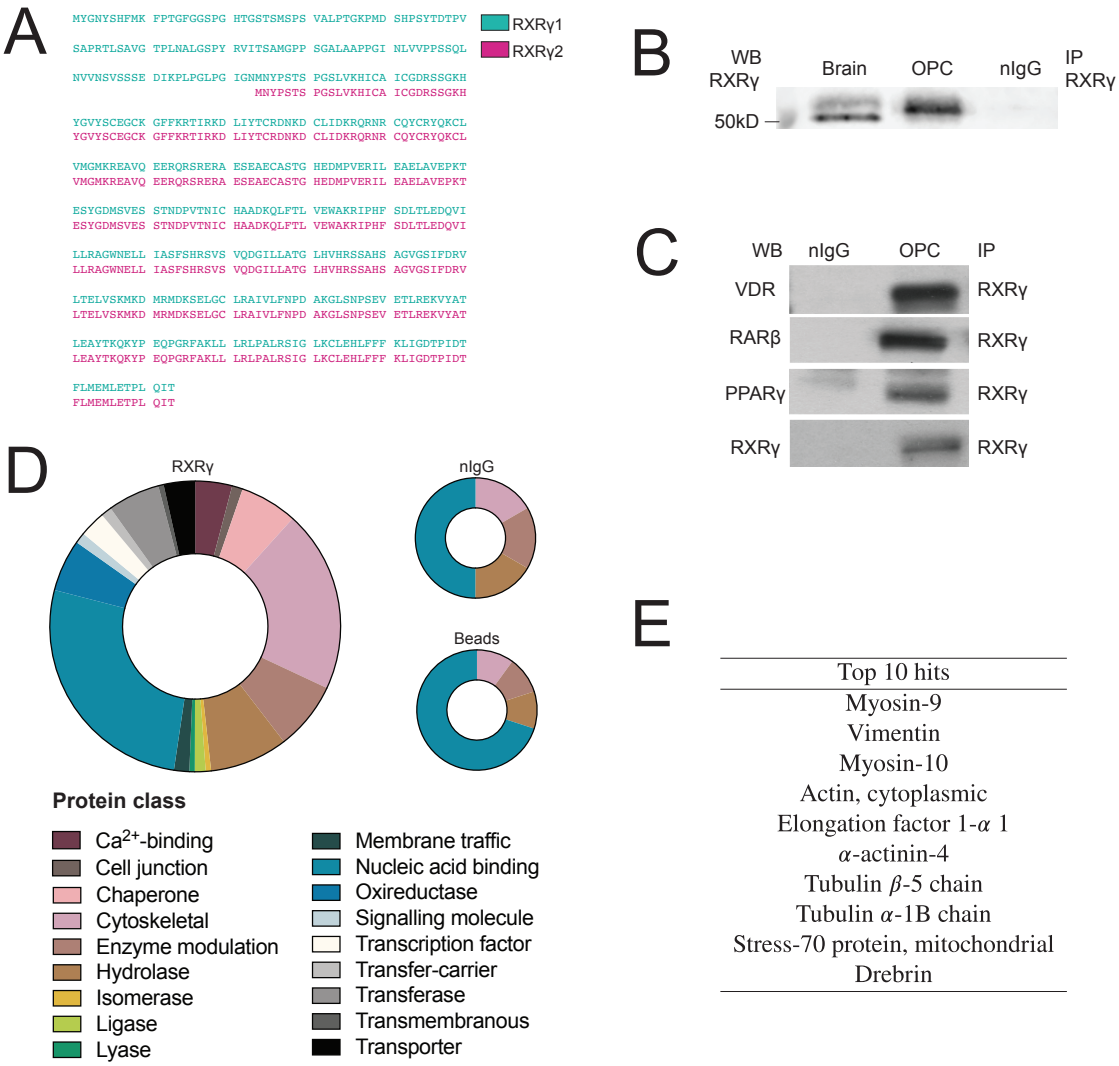
Overall, sorting neonatal rat brain on A2B5 using MACS yields a population of completely viable cells which are highly enriched in OPCs. This population was then lysed immediately without prior *in vitro* culturing, and CoIP for RXRγ was carried out.

3.1.2 RXR CoIP and mass spectrometry analysis

RXRγ has been reported to have two splice variants: RXRγ1 and RXRγ2 (Liu, 1993; Seleiro et al., 1994). The alignment of the protein sequences of RXRγ1 and 2 is shown Figure 3.2A. The overall molecular weight (Mw) of the larger isoform, RXRγ1, is 50.9 kilodaltons (kD), and by subtracting the Mw of the extra amino acids, RXRγ2 results in a Mw of 38.6kD. Previous characterisation of RXRγ expression in the oligodendrocyte lineage was carried out in the Franklin laboratory, and revealed that both RXRγ splice variants are expressed in OPCs and oligodendrocytes (de la Fuente, 2014).

In order to ensure that the CoIP method used was pulling down RXRγ, western blots of the eluted solution were run and stained for RXRγ. Figure 3.2B shows that the RXRγ CoIP results in the pull down of RXRγ1, as the band obtained is approximately 50kD in size. This result is observed in both OPC and whole brain lysate. Furthermore, previous validation of this CoIP method by Alerie Guzmán de la Fuente demonstrated that partners reported to heterodimerise with RXRγ in the literature are also pulled down when carrying out CoIP with RXRγ, as shown in the western blots in Figure 3.2C, suggesting that this method, in combination with an appropriate antibody, efficiently pulls down RXRγ complexes (de la Fuente, 2014).

The CoIP elution was taken to GlaxoSmithKline for mass spectrometry, which was kindly carried out by Carla Newman. Mass spectrometry analysis revealed no presence of RXRγ, other NRs or co-regulators. The table in Figure 3.2E shows that the top 10 unique peptide sequences detected by the mass spectrometer belong to proteins involved in organising the cytoskeletal structure of cells. Further analysis of the results was carried out using the PANTHER database (http://www.pantherdb.org) for protein class analysis. This clustered all the proteins detected into similar classes as summarised by the donut graph in Figure 3.2D. The nucleic acid binding class is composed of ribosomal proteins, histones and mRNA-binding proteins. The transcription factor class is one of the most interesting due



Top 10 hits
Myosin-9
Vimentin
Myosin-10
Actin, cytoplasmic
Elongation factor 1-α 1
α-actinin-4
Tubulin β-5 chain
Tubulin α-1B chain
Stress-70 protein, mitochondrial
Drebrin

Fig. 3.2 Mass spectrometry analysis of RXRγ CoIP. (A) Protein alignment of the two RXRγ splice variants: RXRγ1 and RXRγ2. RXRγ2 lacks the initial N terminal sequence of RXRγ1, resulting in a Mw of 12.3kD less. (B) Western blot of RXRγ pull down via CoIP. The band obtained weighs approximately 50kD, matching the Mw of RXRγ1. (C) Further validation of the CoIP had been previously carried out by assessing the pull down of partners known to associate to RXRγ. (D) Donut graph summarising the proteins in the RXRγ CoIP elution identified by mass spectrometry. The proteins were clustered by protein class. The majority of proteins identified belong to the nucleic acid binding class and cytoskeletal class, containing 26.7% and 20.3% of the total proteins identified respectively. No RXRγ, NR partners or co-regulators were detected. In the normal IgG control and beads-only control, less proteins were detected, however, extensive detection of nucleic acid binding and cytoskeletal proteins still occurred, suggesting they could be pulled down due to "stickiness" of the resin beads. (E) Table presenting the top 10 proteins detected by mass spectrometry. The results shown are the number of times a set of *unique* peptide sequences were detected, therefore this is not a fully quantitative method. Consequently the table is not a ranking of the most abundant peptides in the sample.

to the way in which NRs signal. However, only five proteins were clustered in this class. Data is shown for the RXRγ antibody which allowed the detection of the highest number of peptides (Abcam, rabbit anti-RXRγ, cat# ab15518). In the corresponding nIgG control and beads-only control, significantly fewer peptides were detected, 96.5% and 93.5% less compared to the RXRγ sample. Thus, it shows that this antibody in particular may be pulling down non-specific proteins. Other RXRγ antibodies pulled down fewer peptides than RXRγ ab15518. However, RXRγ, its NR partners and co-regulators were not detected with the use of other antibodies either.

3.1.3 Troubleshooting the mass spectrometry analysis of RXR complexes

Neither NR partners nor co-regulators were detected. However, the most unexpected finding was the absence of RXRγ from the above analysis, as the CoIP is designed to enrich the eluted solution for the protein being pulled down. To troubleshoot this issue, trials using whole brain lysates were initially used to try and increase the yield of RXRγ. Following this, nuclear versus cytoplasmic lysis was carried out and only the nuclear lysate was used for CoIP to enrich for NRs. However, neither attempts yielded successful results in that RXRγ was not detected by the mass spectrometer in either conditions. Three different RXRγ antibodies were used for CoIP in case the specific antibody being used was not pulling down enough RXRγ. The resin beads used in the CoIP were also replaced with magnetic beads which are less "sticky" to non-specific proteins, in order to limit contamination of proteins known to be highly expressed in all cells, such as cytoskeletal proteins, but without success. An attempt with no CoIP was carried out in order to avoid lysate processing and to elucidate whether NRs could be simply picked up the the mass spectrometer: however, this also failed. All of the above results are not presented in this thesis.

After the above attempts, there could be two main reasons as to why RXRγ and its partners are not picked up via mass spectrometry:

1. the antibodies used to target RXRγ are not specific enough, resulting in the pull down of contaminants;

2. the expression of NRs is too low to be detected by the mass spectrometer used.

To assess whether the antibodies used were binding to RXRγ, I carried out western blot on recombinant RXRγ as shown in Figure 3.3A. This shows that all four antibodies used for CoIP do indeed bind RXRγ. Whilst this does not exclude the possibility of the non-specific binding of these antibodies to other proteins, it gives confidence that all of them should be

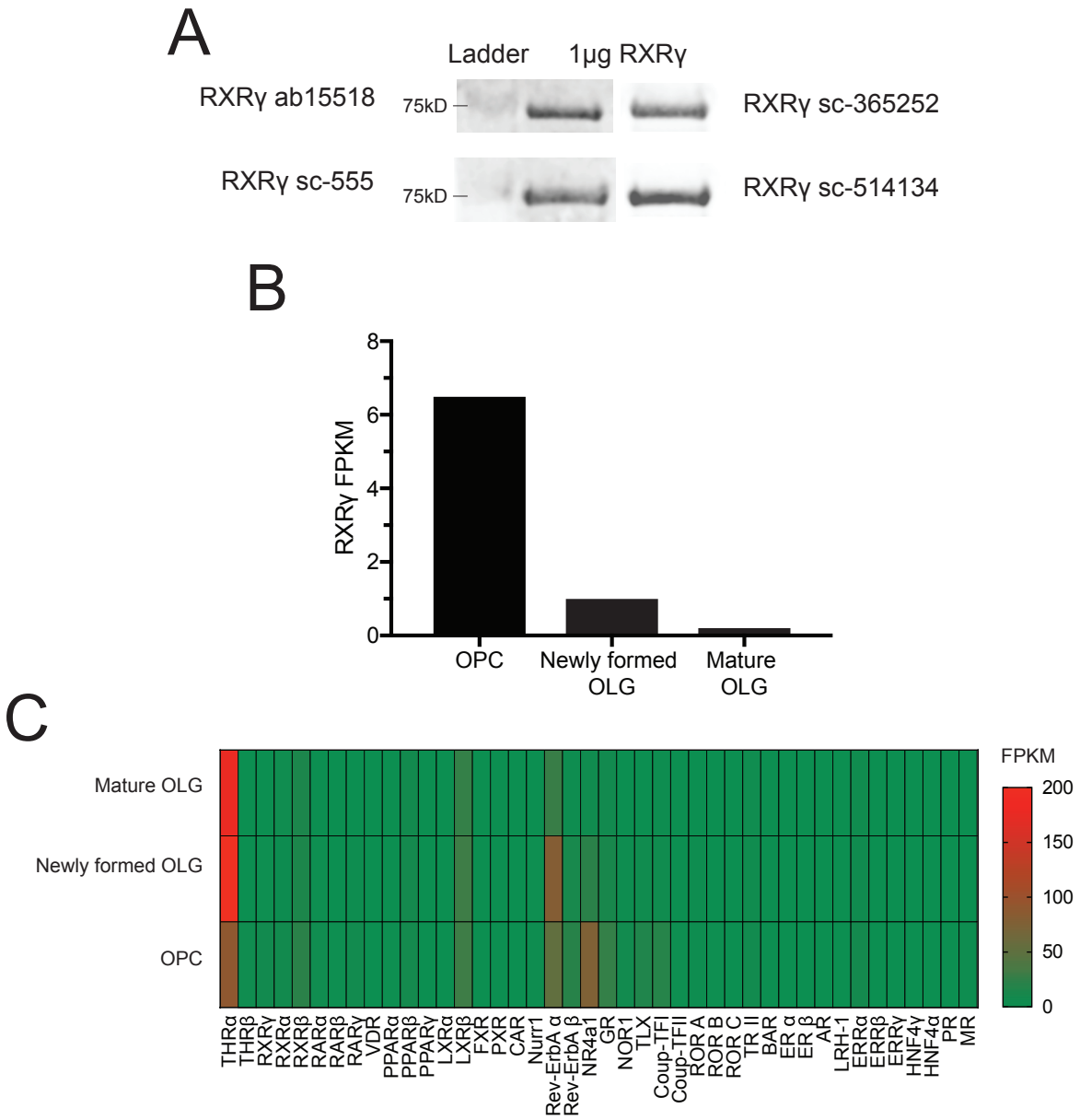


Fig. 3.3 Mass spectrometry troubleshooting. (A) Western blots run using recombinant GST-tagged RXRγ. Four different RXRγ antibodies were tested and all four show binding to RXRγ. RXRγ here presents a 75kD Mw as it is GST-tagged. (B) The use of the brain RNA-seq database created by Zhang et al. (2014) shows the low expression level of RXRγ in oligodendrocyte lineage cells. Interestingly, when comparing the differential expression of genes between the three stages of the lineage, RXRγ is in the top differentially expressed genes in OPCs compared to the other two stages. (C) A heat-map generated with the Zhang et al. (2014) database. This shows that the vast majority of NRs are expressed at extremely low levels in oligodendrocyte lineage cells, hence the reason why mass spectrometry was unable to detect any NRs despite the enrichment in RXRγ through CoIP.

pulling down RXRγ. In order to appropriately assess non-specific binding, a protein lysate without RXRγ should be included as the most appropriate negative control. However, as NRs are ubiquitously expressed, it is not possible to obtain this from any rat tissue.

When the lysates were spiked with recombinant RXR, this was detected by the mass spectrometer, leading me to suspect that the expression of RXRγ is below the detection limit of the mass spectrometer. Indeed, when plotting the expression of RXRγ in oligodendrocyte lineage cells using the results obtained from the RNA-seq database created by Zhang et al. (2014), it is clear that RXRγ is expressed at extremely low levels, with 6.5 FPKM being the highest amount observed in OPCs (Figure 3.3B). Likewise, other NRs present extremely low expression levels, in the majority of cases close to 0 FPKM, as summarised by the heat-map in Figure 3.3C.

3.1.4 Discussion

I have presented data indicating that the major unique signature peptides detected by mass spectrometry upon RXRγ CoIP belong to cytoskeletal and nucleic acid binding proteins. Due to the absence of RXRγ amongst the detected peptides, I believe that these results do not provide a clear answer on the list of proteins forming RXRγ complexes in OPCs. This is due to the unspecific pull down of proteins, such as cytoskeletal ones, and/or RXRγ expression levels being below the detection limit of the mass spectrometer. In order to assess the specificity of the RXRγ antibodies it is necessary to have an appropriate control not expressing any RXRγ. For this purpose, western blot using cells isolated from RXRγ knockout mice could have been carried out. However, such mice would have needed to be re-derived as none are currently available to the lab. Alternatively, RXRγ could have been knocked out *in vitro* using CRISPR, and for this purpose any cell type, including cell lines, could have been used. However, OPC and whole brain western blot using the RXRγ antibody reported, results in the detection of RXRγ1 (Figure 3.2B), raising the question of whether it is the antibody that is binding the array of proteins reported by mass spectrometry.

Nonetheless, it has been reported that cytoskeletal proteins are able to influence gene transcription. This is reasonable as cells need to be able to respond to mechanical cues in order to match and react to the physical demands of their environment (Discher et al., 2005). Indeed, mechanobiological studies have highlighted the influence that mechanical stressors have on an array of cellular functions, including apoptosis, migration, proliferation, differentiation and lineage specification (Wang et al., 2000; Engler et al., 2006; Klein et al., 2009; Raab et al., 2012). These processes require tight transcriptional regulation and it has been suggested that the mechanotransduction is transferred from cytoskeletal proteins such as actin to the nuclear lamina, whose components are able to influence transcription factors and

can directly associate to the DNA (Stierlé et al., 2003; Malhas et al., 2009; Simon et al., 2010). Cytoskeletal proteins such as actin have also been reported to be components of chromatin remodelling complexes and to interact directly with NRs. Nuclear β-actin and its binding proteins are components of the SWI/SNF family of chromatin remodelling complexes (Shen et al., 2003; Miralles and Visa, 2006), known to be recruited by NRs. In oligodendrocyte lineage cells, SWI/SNF promotes the expression of myelin genes via the lineage-specific transcription factor Sox10 (Dilworth et al., 2000; Marathe et al., 2013). Furthermore, actin binding proteins have been reported to regulate numerous class I and III NRs as reviewed by Gettemans et al. (2005). This phenomenon has been exceptionally studied in AR signalling, where actin binding proteins such as gelsolin, supervillin and filamin have been shown to act as co-regulators (Loy et al., 2003; Nishimura et al., 2003; Ting, 2004). This information points towards the possibility of a direct involvement of cytoskeletal proteins such as actin and vimentin in the formation of NR complexes, therefore, the pull down of these proteins by RXRγ and their detection via mass spectrometry may not be an artefact but rather a true result reflecting the complicated function and signalling of RXR heterodimers. Whilst NRs have conventionally been described as transcription factors governing gene expression, RXR and other NRs also present genomic-independent functions. These vary from phosphorylation control by regulating the activity of enzymes such as kinases and phosphatases, regulation of intracellular Ca^{2+} levels and control of ion channel function to name a few (Boonyaratanakornkit et al., 2001; Skildum et al., 2005; Hammes and Levin, 2007; Sarkar et al., 2008; Ordóñez-Morán et al., 2008). RXR, and some of its partners such as PPAR, have also been found to localise in the mitochondria, where they are believed to control transcription of mitochondrial DNA (Casas et al., 2003; Lin et al., 2008; Chang and Ha, 2018). Furthermore, in order for RXR to be translocated from the cytoplasm to the nucleus and *vice versa*, its association to transporters is essential. To summarise, the array of proteins detected by the mass spectrometer may not necessarily be due to unspecific protein binding or contamination of the preparation via other means. It rather may reflect the complex biochemical picture presented by a receptor which interacts with a plethora of different protein classes due to both its genomic and non-genomic functions, as well as all those interactions involved in correct receptor assembly, structure and localisation.

Despite this, the inability to detect RXRγ, its partners and co-regulators raises concerns on whether the data is a true reflection of RXRγ protein complexes. The heat-map in Figure 3.3C shows the excessively low expression of NRs in oligodendrocyte lineage cells. This could be a limiting factor that would compromise the detection of NRs despite their enrichment via CoIP, but the proteins detected would still be trustworthy. The extremely low expression level of NRs does not come as a surprise, as these receptors are highly specific

to their function and, therefore, a small amount may be enough to elicit the desired effects. Additionally, limiting the pool of RXR and other NRs, is a strategy for tightly regulating their activation and signalling, supporting the necessity for keeping their expression at low levels as described in Section 1.5.2.

Spiking the sample taken to mass spectrometry with recombinant RXR allowed for its detection (results not shown). In order to troubleshoot the low expression level I decided to overexpress RXRγ in primary rat OPCs in order to see whether that would allow for RXRγ detection. For this purpose I designed a FLAG-tagged RXRγ vector. This would allow both the increase of RXRγ expression and the use of a highly specific antibody for the CoIP thanks to the FLAG tag, thereby troubleshooting both issues. Unfortunately transfection of primary OPCs with the vector resulted in excessive cell death and low levels of transfection (data not shown). For this reason I decided to use a biased approach based on the literature, in order to test the partner switching hypothesis. This will be further discussed in Section 3.2.4.

3.2 Levels of control for RXR-NR signalling in oligodendrocyte lineage cells

3.2.1 RXR activation in serum-free conditions

In Section 3.1 I have shown that carrying out CoIP on RXRγ resulted in no detection of known NR partners by mass spectrometry. Despite the conclusion of this section, a fundamental question remains: if no partners are detected, does RXRγ really promote OPC differentiation via heterodimerisation, or could the effects observed by Huang et al. (2010a) occur via homodimeric or monomeric RXRγ signalling?

In order to answer this question, one must be able to observe the *true* effect of RXR activation without the stimulation of other NRs. All previous experiments have been carried out using mixed glia isolated OPCs. As explained in Section 2.2.2, this technique exposes the culture to serum for 10 days before isolating the OPCs from other brain cells. Ligands for NRs include hormones, vitamins and retinoids, which circulate the body via blood plasma. Therefore, the serum used in culture is abundant in NR ligands (Chen et al., 1997; Dang and Lowik, 2005; Rauch, 2011). Consequently, the NR data presented by studies using mixed glia isolation may be influenced by the chronic exposure of OPCs to NR ligands, able to activate both RXR and its partners. Thus, the effects observed would not result from the individual treatment carried out, but rather from the overall effect of the individual treatment combined with the cell's prior exposure to an array of different NR ligands. The limits of serum when assessing NR biology have been observed in past studies, where the use of mixed glia cultures impaired the observation of the effects elicited by calcitriol on OPCs, as serum exposure resulted in the saturation of the system (de la Fuente et al., 2015). Serum stripping using charcoal solved the above issue; this involves incubating serum with activated carbon which binds to and removes all steroid-like molecules such as NR ligands (de la Fuente et al., 2015). The removal of lipophilic ligands creates a more defined serum with a lower chance of influencing the experimental results.

However, charcoal stripping does not completely remove all forms of lipophilic molecules, and the extent of the stripping may vary due to both the initial composition of different batches of serum and technical inconsistencies. Due to this, I decided to carry out NR ligand *in vitro* treatments on MACS isolated OPCs. As the cells are acutely isolated from the brain and plated directly as a pure OPC population in serum-free media, serum and NR ligand exposure is avoided and the effect of individual receptor activation on OPCs can be observed.

3.2.2 Purity of cultured OPCs isolated via MACS

The purity of acutely isolated OPCs has been previously assessed in Figure 3.1. However, OPCs are able to form type II astrocytes *in vitro* (Raff et al., 1984), and highly proliferative cells such as microglia and pericytes can result in their taking over the surface area available for culture. It is therefore important to know how the OPC culture evolves over time in order to ensure a reliable culture system throughout the experiment.

As shown in Figure 3.4A, the density at which OPCs are plated after MACS influences astrocyte formation over time. The panel is a qualitative representation of the amount of astrocytes observed after eight days of *in vitro* culture. OPCs were plated at three different densities immediately after MACS isolation. At lower densities less OPCs differentiate into astrocytes: this is important as an accumulation of astrocytes prevents OPC differentiation into oligodendrocytes, potentially due to the release of GFs that maintain OPCs in a proliferative state (Raff et al., 1988; Messersmith et al., 2000; Ito et al., 2005; Lu et al., 2009; Cabezas et al., 2016). To avoid this, all MACS isolated OPCs were plated at a density of 6250 OPCs/cm^2.

Figure 3.4B, shows that when plated at this density, the percentage of cells expressing Olig2 is on average 77.77% (SD=17.60, n=3) 4 hours after plating, and remains constant as there is no significant difference in the percentage of Olig2 expressing nuclei over the course of 72 hours in the absence of GFs (ordinary one-way ANOVA, p=0.3660, n=3). Of this Olig2 population, no cells express the mature oligodendrocyte marker MBP and this does not change in the first three days of culture (Kruskal-Wallis, p>0.999, n=3), whilst an average of 62.48% (SD=7.47, n=3) of Olig2 expressing cells also express the OPC marker NG2 (Figure 3.4C). This significantly increases over time to an average of 72.76% (SD=5.34, n=3) (ordinary one-way ANOVA, p=0.0047**, n=3). Figure 3.4C also shows that no astrocytes were acutely isolated via MACS, and when plated at low densities no astrocytes form in the first three days in culture (Kruskal-Wallis, p=0.2088, n=3). Microglia do not seem to proliferate as there is no significant increase in the number of CD11b expressing cells after 72 hours (ordinary one-way ANOVA, p=0.9283, n=3). A percentage of cells (on average 16.48%, SD=7.47, n=3) expresses NG2 but not Olig2. These cells may be pericytes and endothelial cells as they are also known to express NG2 (Pouly et al., 2001; Guimarães-Camboa et al., 2017). However, these cell types are highly proliferative and three days after plating there is no significant difference in the amount of NG2-only expressing cells (ordinary one-way ANOVA, p=0.2207, n=3). If the NG2-only cells are part of the oligodendrocyte lineage but do not express Olig2 due to excessive stress, this percentage is expected to decrease over time. Indeed, the percentage of these cells in culture does decrease to an average of 0.44%

(SD=0.505, n=3) 72 hours after MACS isolation; however, this does not translate to a statistically significant difference, leaving these cells unidentified.

A small percentage of DAPI cells does not express any of the assessed markers after 4 hours (on average 5.75%, SD=3.45, n=3). The number does not change significantly over time (ordinary one-way ANOVA, p=0.3351, n=3).

According to flow cytometry, immediately after MACS isolation almost all cells isolated are viable and 87.47% of the cells are OPCs (Figure 3.1). However, MACS isolation induces shear stress on the isolated cells due to mechanical tissue dissociation and the passing through the column. Based on the above, it is probable that the majority of cells plated are indeed OPCs: however, initial cell death and cell recovery compromise the purity in the first hours and days following the isolation, as stressed cells may not express markers at a level detectable by immunocytochemistry.

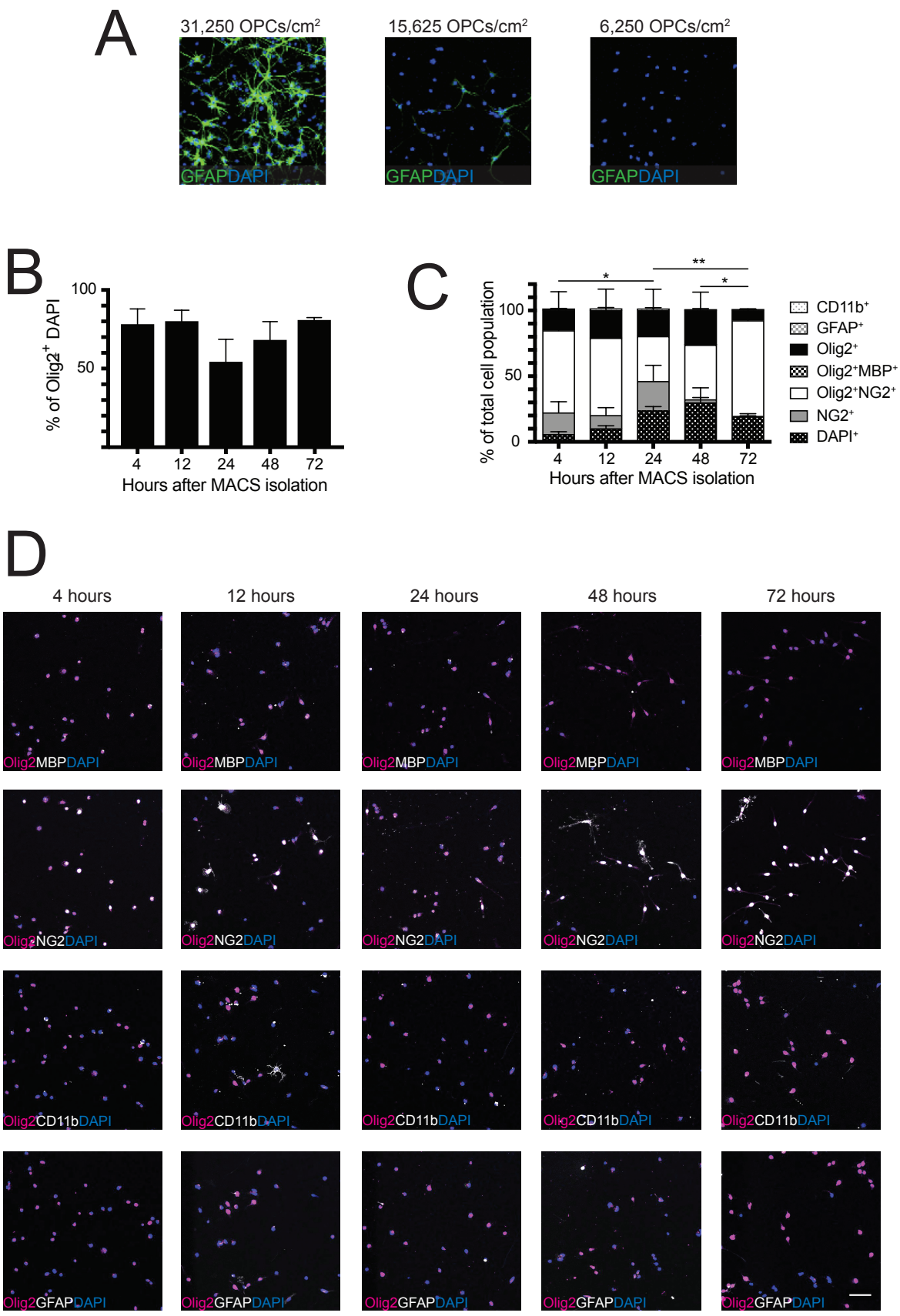

A
31,250 OPCs/cm²
15,625 OPCs/cm²
6,250 OPCs/cm²
GFAPDAPI
B
% of Olig2⁺ DAPI
100
50
4 12 24 48 72
Hours after MACS isolation
C
% of total cell population
100
50
0
*
**
*
4 12 24 48 72
Hours after MACS isolation
CD11b⁺
GFAP⁺
Olig2⁺
Olig2⁺MBP⁺
Olig2⁺NG2⁺
NG2⁺
DAPI⁺
D
4 hours
12 hours
24 hours
48 hours
72 hours
Olig2MBPDAPI
Olig2NG2DAPI
Olig2CD11bDAPI
Olig2GFAPDAPI

Fig. 3.4 Purity of primary rat OPCs extracted by MACS and cultured in serum free conditions. (A) Qualitative immunocytochemistry of type II astrocyte formation based on the seeding density of OPCs. The higher the initial OPC density, the more astrocytes are formed over the course of eight days in culture. Astrocytes are identified using the marker GFAP. Upon determining the initial OPC seeding density of 6,250 OPCs/cm^2, changes in the expression of the lineage marker Olig2 were determined as shown in (B). OPCs were MACS sorted and kept for 72 hours in the absence of GFs. The number of $Olig2^+$ nuclei is on average 77.77% ± 17.6% 4 hours after seeding. The amount of $Olig2^+$ nuclei does not change significantly over the course of the first 72 hours in culture. (C) Of all the $Olig2^+$ population, none express the mature oligodendrocyte marker MBP and this does not change over time. Instead, 62.48% ± 7.471% also express NG2. The amount of $Olig2^+NG2^+$ cells increases significantly to 72.76% ± 5.34% after 72 hours (p=0.0047). The significance of multiple comparisons for the $Olig2^+NG2^+$ is represented in the graph (*p≤0.05 **p≤0.01). Other cell types such as astrocytes (GFAP), microglia (CD11b), NG2 only and DAPI only cells were present in low percentages 4 hours after plating and did not change significantly over the course of the first three days of culture (0.38% ± 0.48% for GFAP, 0.87% ± 0.88% for CD11b, 16.48% ± 14.45% for NG2 only and 5.75% ± 3.45% for DAPI only cells). (D) Representative pictures for each cell type reported in the purity graphs at each time point analysed. Scale bar = 50μm. All data was analysed using ordinary one-way ANOVA followed by Tukey's multiple comparison test where significance was reached or Kruskal-Wallis test where the data did not follow a normal distribution. All graphs are presented as mean ± SEM, biological n=3.

3.2.3 RXR activation alone is not sufficient to promote OPC differentiation

After MACS isolation, OPCs were plated and left to recover for three days in the presence of GFs, after which GFs were removed and OPC media with the desired treatment was added.

The media was replenished every other day and OPCs were chronically exposed to the treatment for eight days. The cells were fixed and stained for the lineage marker Olig2 and the differentiation marker CNPase.

Unlike what has been previously reported in the mixed glia experiments carried out by Huang et al. (2010a), RXR activation via treatment of its agonist 9cRA resulted in no significant difference in the percentage of differentiated cells compared to the vehicle control DMSO after eight days of exposure as shown in Figure 3.6A (ordinary one-way ANOVA followed by Dunnett's multiple comparison test, p=0.8197, n=4). Figures 3.6A and B show that this observation was true for the treatment of all NR agonists and antagonists, except for the THR agonist T3, readily added to the the media of MACS sorted OPCs to promote differentiation and therefore used in this experiment as a positive control (Barres et al., 1994; Baas et al., 1997) (ordinary one-way ANOVA followed by Dunnett's multiple comparison test, p=0.0024, n=4). None of the treatments reported more cell death than the vehicle control as shown in Figure 3.5.

Based on the data presented it appears that the presence of a multitude of NR ligands is important in order to promote OPC differentiation. This is immediately clear when comparing the spontaneous differentiation rate of MACS neonatal OPCs eight days after GF withdrawal to the differentiation rate of mixed glia isolated OPCs after only two days of GF removal (Figures 3.7A and B).

In this experiment the vehicle control presents an average differentiation rate of 1.55% (SD=1.34, n=4) for MACS isolated OPCs eight days after GF withdrawal (Figure 3.7B). When this is compared to mixed glia isolated OPCs exposed to either normal serum or charcoal-treated serum 48 hours after the shake off (average=75%, SD=7.037 and average=61.17%, SD=0.4124 respectively), it is clear that there is a substantial difference in the spontaneous differentiation rate of the same cells (ordinary one-way ANOVA followed by Tukey's multiple comparison test, p<0.0001, n=3). Additionally, there is a significant difference in the differentiation rate of OPCs cultured with normal or charcoal-treated serum, further suggesting the importance of steroid-like molecules in OPC differentiation (ordinary one-way ANOVA followed by Tukey's multiple comparison test, p=0.0130, n=3).

Therefore, activation of RXR alone via its agonist 9cRA is not sufficient to promote OPC differentiation. The previously reported effects of 9cRA were observed in experiments involving mixed glia isolated OPCs, whereby the cells were exposed to serum which contains

other partner ligands. Indeed, stripping serum of such ligands using charcoal also abrogates OPC differentiation induced by 9cRA (de la Fuente et al., 2015). The data presented suggests that RXR does not promote OPC differentiation as a monomer or homodimer, as the presence of other NR ligands is required for OPC differentiation to occur. This is further supported by unpublished experiments carried out by Oihana Errea from the Franklin laboratory shown in Figure 3.7C, where the treatment of MACS isolated OPCs with a combination of ligands such as 9cRA and T3 resulted in an increased differentiation than the individual ligand treatments.

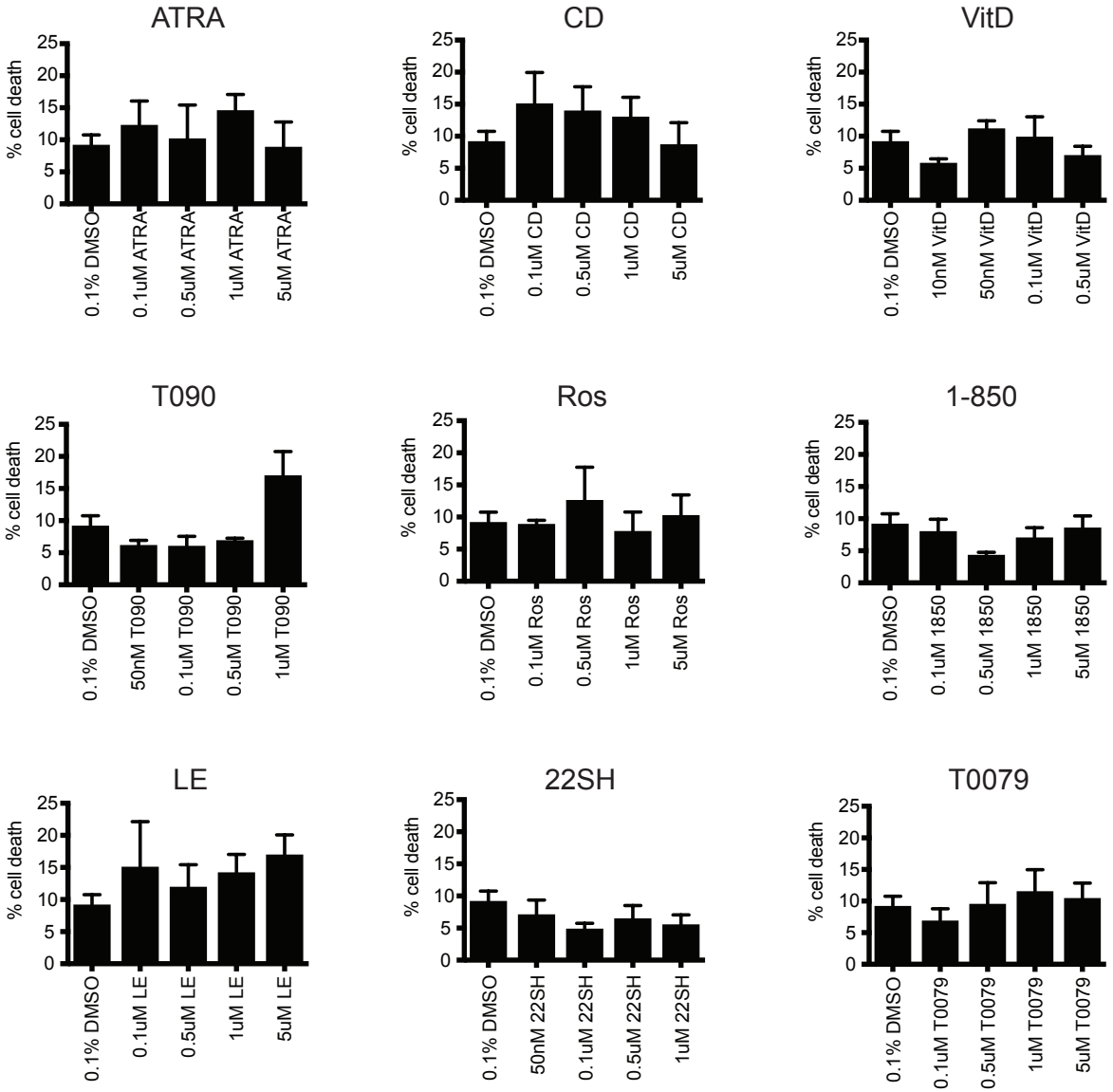
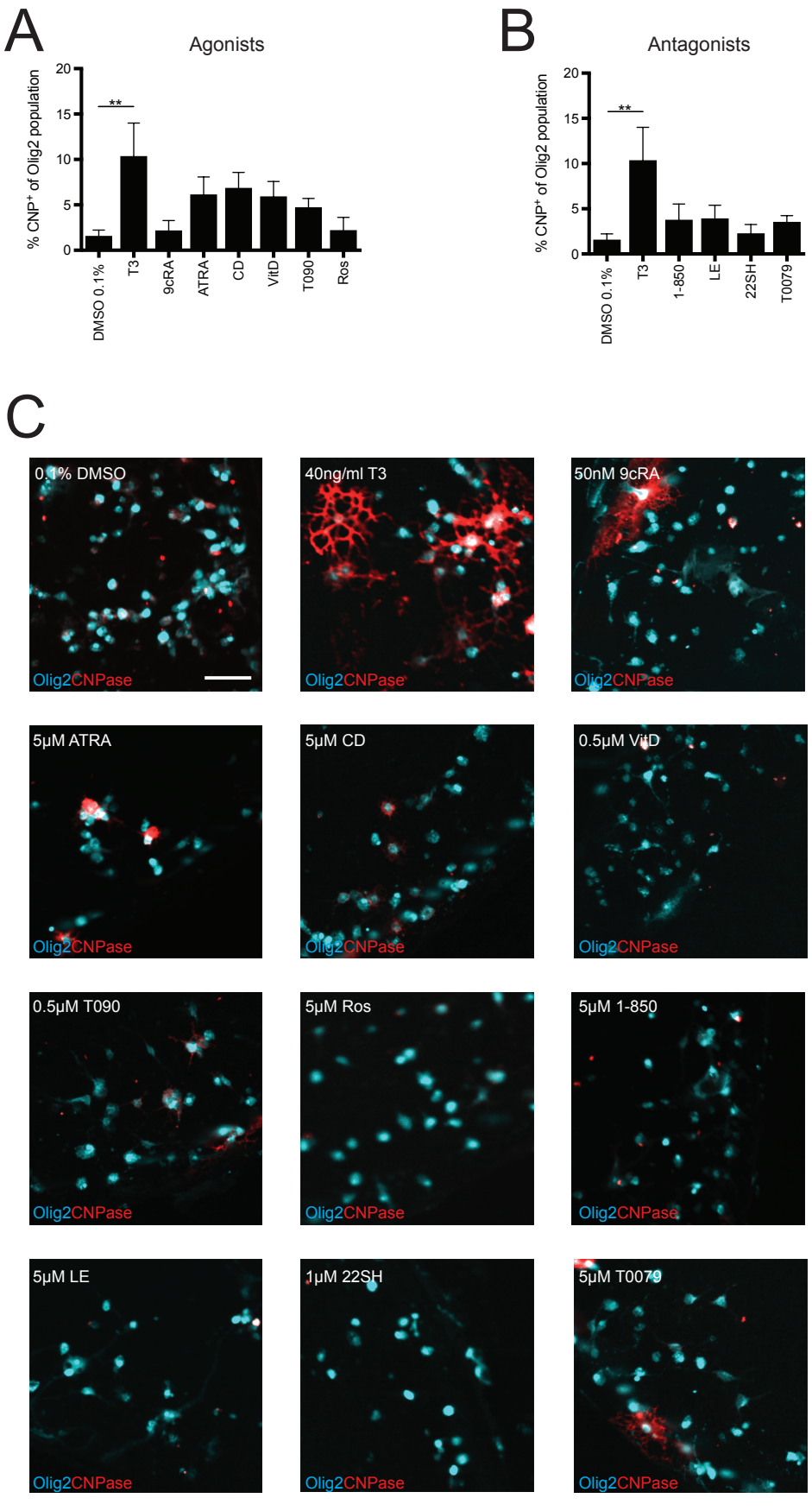

ATRA
% cell death
0
5
10
15
20
25
0.1% DMSO
0.1uM ATRA
0.5uM ATRA
1uM ATRA
5uM ATRA
CD
% cell death
0.1% DMSO
0.1uM CD
0.5uM CD
1uM CD
5uM CD
VitD
% cell death
0.1% DMSO
10nM VitD
50nM VitD
0.1uM VitD
0.5uM VitD
T090
% cell death
0.1% DMSO
50nM T090
0.1uM T090
0.5uM T090
1uM T090
Ros
% cell death
0.1% DMSO
0.1uM Ros
0.5uM Ros
1uM Ros
5uM Ros
1-850
% cell death
0.1% DMSO
0.1uM 1850
0.5uM 1850
1uM 1850
5uM 1850
LE
% cell death
0.1% DMSO
0.1uM LE
0.5uM LE
1uM LE
5uM LE
22SH
% cell death
0.1% DMSO
50nM 22SH
0.1uM 22SH
0.5uM 22SH
1uM 22SH
T0079
% cell death
0.1% DMSO
0.1uM T0079
0.5uM T0079
1uM T0079
5uM T0079

Fig. 3.5 Titrations assessing cell death of NR ligand treatments carried out in Figure 3.6. Rat primary OPCs were isolated using MACS and left to recover in GFs in serum-free conditions. After two days GFs were removed and the cells were chronically exposed to the treatments for eight days. The media was changed every other day. Cell death was assessed by the percentage of $DAPI^{+}PI^{+}$ staining. The concentration range picked was determined by what was reported in the literature and the experiments previously carried out in the Franklin laboratory. Abbreviations for the treatments are explained in Figure 3.6D. All data was analysed using ordinary one-way ANOVA. All graphs are presented as mean ± SEM, biological n=3.


A
Agonists
% CNP+ of Olig2 population
20
15
10
5
0
**
DMSO 0.1%
T3
9cRA
ATRA
CD
VitD
T090
Ros
B
Antagonists
% CNP+ of Olig2 population
20
15
10
5
0
**
DMSO 0.1%
T3
1-850
LE
22SH
T0079
C
0.1% DMSO
40ng/ml T3
50nM 9cRA
5μM ATRA
5μM CD
0.5μM VitD
0.5μM T090
5μM Ros
5μM 1-850
5μM LE
1μM 22SH
5μM T0079
Olig2CNPase

D

Name	Abbreviation	Type	NR
Triiodothyronine	T3	Agonist	THR
9-*cis*-retinoic acid	9cRA	Agonist	RXR
All-*trans* retinoic acid	ATRA	Agonist	RAR
CD2314	CD	Agonist	RARβ
1a25-Dihydroxyvitamin D3	VitD	Agonist	VDR
T 0901317	T090	Agonist	LXR
Rosiglitazone	Ros	Agonist	PPAR
1-850	1-850	Antagonist	THR
LE135	LE	Antagonist	RAR
22(S)-Hydroxycholesterol	22SH	Antagonist	LXR
T0070907	T0079	Antagonist	PPAR

Fig. 3.6 NR ligand treatment in serum-free conditions. Primary rat OPCs were isolated via MACS sorting and cultured in serum-free conditions. OPCs were left to recover in the presence of GFs. After two days the GFs were removed and the treatments were added. OPCs were chronically exposed to the treatments for eight days with media changes carried out every other day. Differentiation was assessed by determining the percentage of CNPase$^+$ cells out of the Olig2$^+$ population. Both (A) agonists and (B) antagonists for NRs show no effect on the differentiation of MACS isolated OPCs apart from the activation of THR via T3, here used as positive control. These treatment conditions present the same cell death as the vehicle control as shown in Figure 3.5. (C) Panel showing the representative immunocytochemistry pictures of NR ligand treatment in serum-free conditions. (D) Table showing the full name of the ligands used in this experiment, which NR they act on and whether they activate or prevent their action. All data was analysed using ordinary one-way ANOVA followed by Dunnett's multiple comparison test where significance was reached (*p$\leq$0.05 **p$\leq$0.01 ****p<0.0001). All graphs are presented as mean $\pm$ SEM, biological n=3. Scale bar = 50μm.

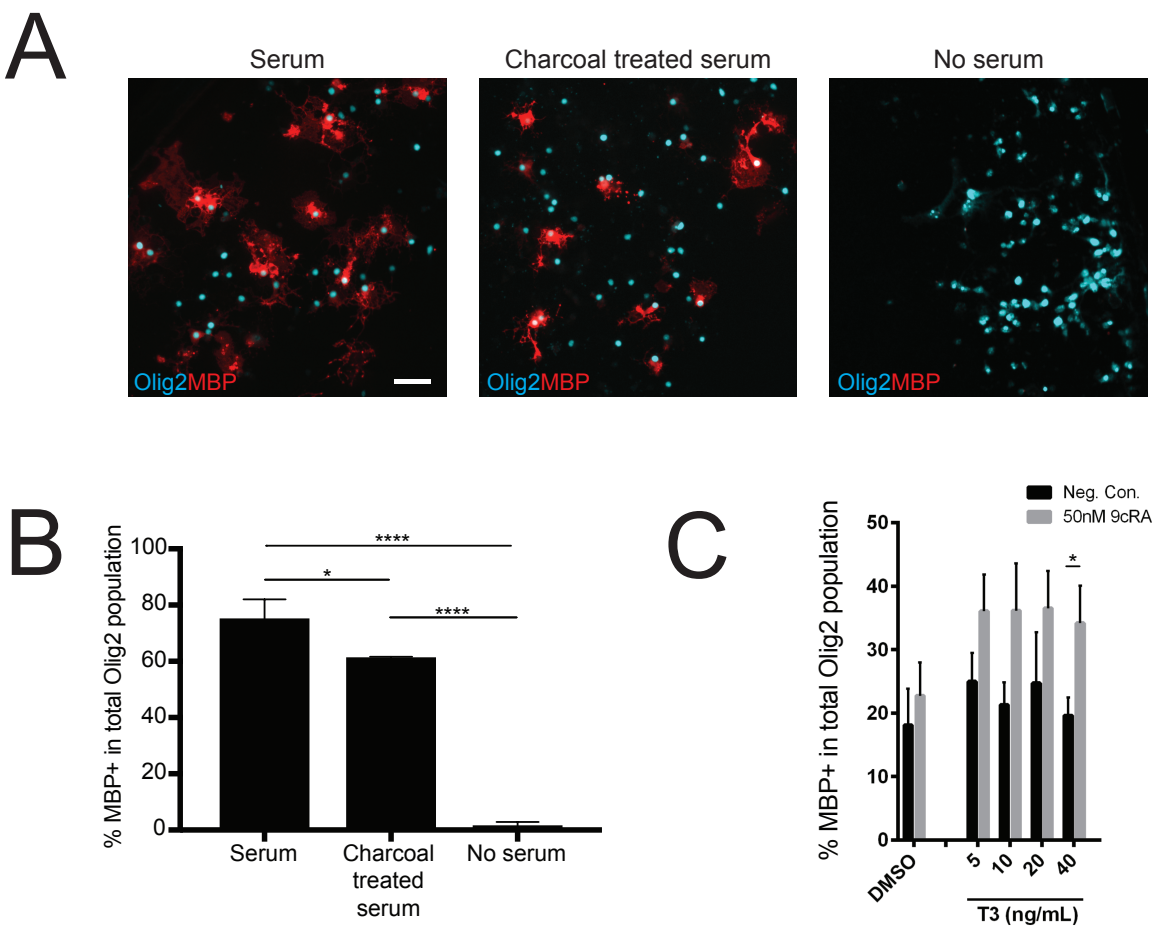

A
Serum
Charcoal treated serum
No serum
Olig2MBP
Olig2MBP
Olig2MBP
B
% MBP+ in total Olig2 population
100
80
60
40
20
0
****
*
****
Serum
Charcoal treated serum
No serum
C
Neg. Con.
50nM 9cRA
% MBP+ in total Olig2 population
50
40
30
20
10
0
*
DMSO
5
10
20
40
T3 (ng/mL)

Fig. 3.7 Spontaneous OPC differentiation after normal serum, charcoal-treated serum and serum-free media exposure. Primary rat OPCs were isolated via mixed glia isolation and kept in normal or charcoal-treated serum before shake off, or via MACS and kept in serum-free conditions. The mixed glia isolated OPCs were left to differentiate for 48 hours, whilst the MACS isolated OPCs were left to differentiate for eight days. Differentiation was assessed based on the percentage of MBP^+ cells out of the whole $Olig2^+$ population. (A) Panel showing the representative immunocytochemistry pictures of spontaneous OPC differentiation after exposure to normal serum, charcoal-treated serum and OPCs in serum-free media. (B) Quantification of (A) showing the comparison of the spontaneous OPC differentiation of OPCs exposed to the three different media conditions. (C) Experiments carried out by Oihana Errea on the differentiation of OPCs sequentially exposed to T3 and 9cRA in serum-free conditions after six days of chronic exposure to the treatments. All data was analysed using ordinary one-way ANOVA followed by Tukey's multiple comparison test where significance was reached (*$p \leq 0.05$ **$p \leq 0.01$ ****$p < 0.0001$). All graphs are presented as mean $\pm$ SEM, biological n=3. Scale bar = 50μm.

3.2.4 RXR is constantly bound to its partners during OPC differentiation

To test the partner switching hypothesis, it is necessary to identify the binding of RXRγ to its partners. As the mass spectrometry based unbiased approach adopted in Section 3.1 did not allow for this, I decided to adopt a biased approach. This consists of carrying out proximity ligation assays between RXRγ and partners previously reported to be involved in OPC biology.

For these experiments I used the Duolink® technique, involving the use of antibodies and DNA technology. If two proteins are considered close enough to be interacting, this translates into a fluorescent signal which can be imaged via conventional fluorescent microscopy as shown in the diagram in Figure 2.3. The advantage is that the Duolink® avoids the use of protein engineering required for FRET, which alters protein structure and can therefore impair the receptor's binding ability.

I used mixed glia cultures in order to have a comparable number of differentiated oligodendrocytes and OPCs for a better comparison between the various differentiation stages. Four different lineage stages were compared as shown in Figure 3.8. OPCs were marked using NG2 and were classified as cycling or non-cycling based on EdU incorporation. Newly formed oligodendrocytes were identified by the absence of MBP and the expression of ectonucleotide pyrophosphatase/phosphodiesterase family member 6 (ENPP6) (Xiao et al., 2016), whilst mature oligodendrocytes were identified by the expression of MBP. The association between RXRγ and any partner was quantified based on Duolink® signal intensity and this was normalised by the area of the nucleus at each of the four stages. Protein quantification via immunofluorescence is usually avoided due to its high variability and lack of reproducibility, and is considered semi-quantitative at best. However, the Duolink® signal has been subject of quantitative image analysis by other studies, highlighting the possibility of its quantification provided that an automated counting method is used (Nilsson et al., 2010).

Figure 3.9A compares the association of RXRγ to each partner at each stage (ordinary one-way ANOVA followed by Tukey's multiple comparison test where there is significance, n=3). In both cycling and non-cycling OPCs the Duolink® signal produced by RXRγ-LXRβ binding is significantly higher compared to any other RXRγ-NR interaction tested ($p<0.0001$). This is observed only at the OPC stage of the lineage, as the later stages present no significant difference in RXRγ association with all the partners assessed. All other partners show no difference in the extent of RXRγ binding for all four stages.

When assessing the binding pattern for each RXRγ-NR individually (Figure 3.9C), it is clear that all partners show a similar pattern of association to RXRγ, where the binding is

strongest at the early stages of the lineage and decreases significantly as OPCs differentiate into mature oligodendrocytes (ordinary one-way ANOVA followed by Tukey's multiple comparison test where there is significance, n=3).

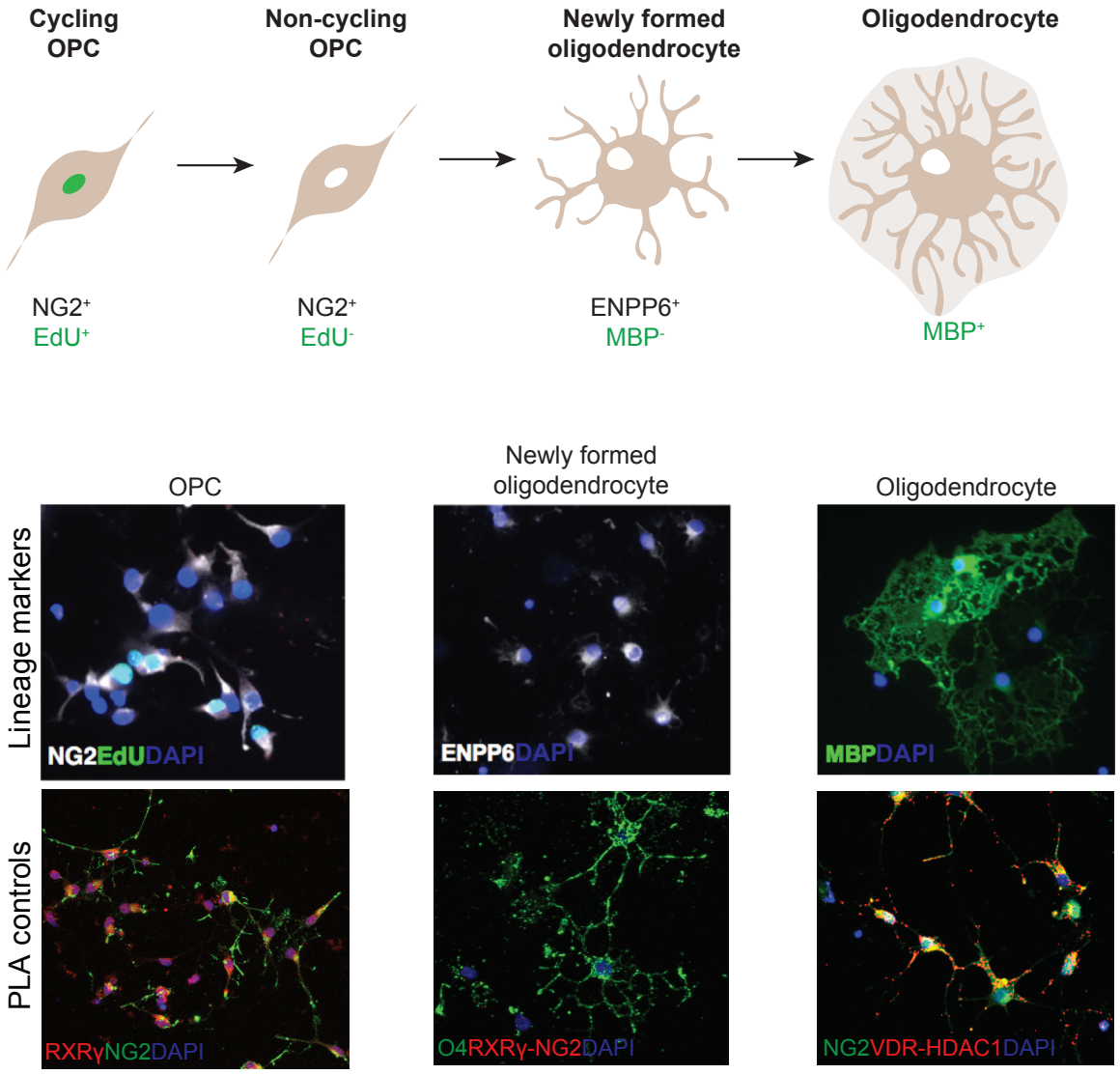
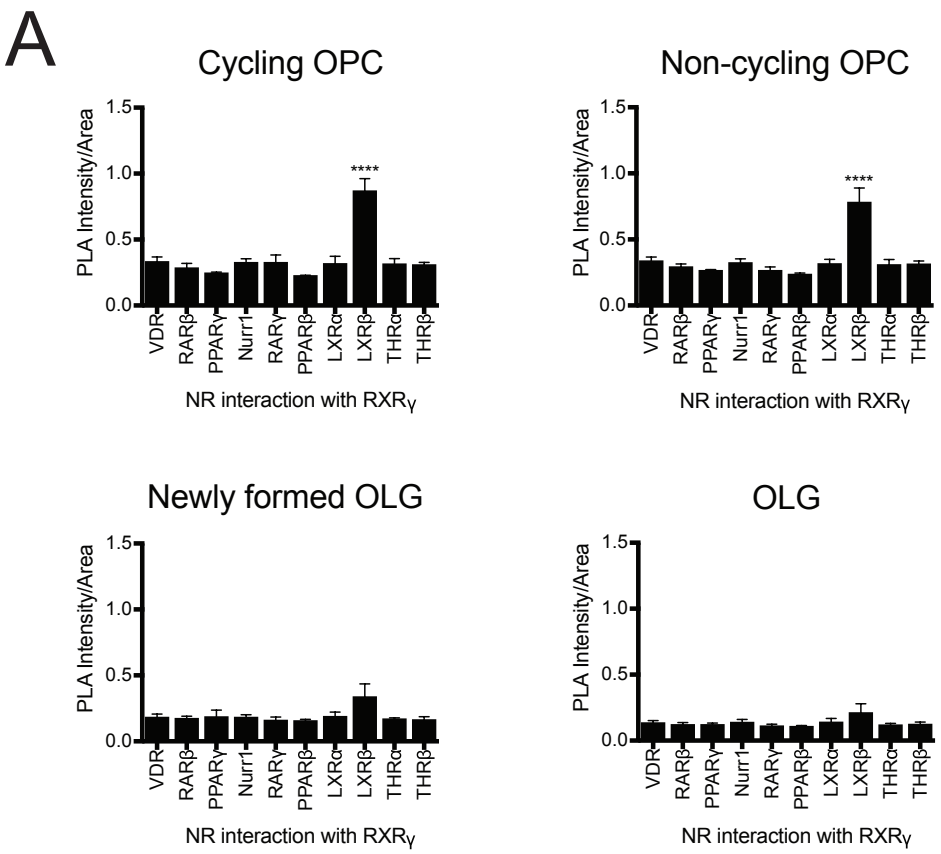
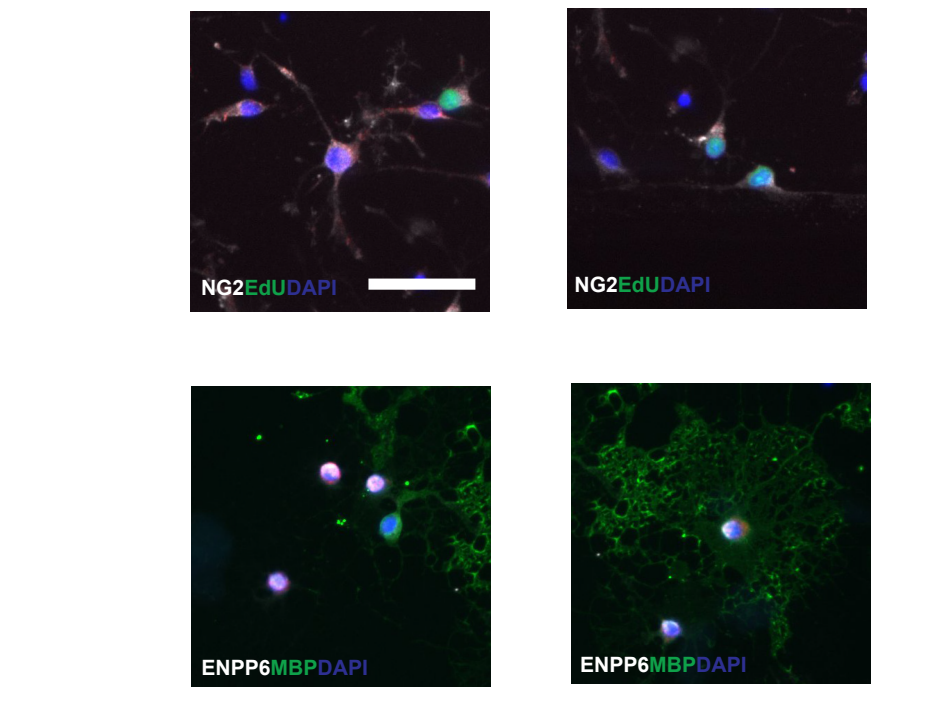

Cycling OPC
Non-cycling OPC
Newly formed oligodendrocyte
Oligodendrocyte
NG2+ EdU+
NG2+ EdU-
ENPP6+ MBP-
MBP+
OPC
Newly formed oligodendrocyte
Oligodendrocyte
Lineage markers
PLA controls
NG2EdUDAPI
ENPP6DAPI
MBPDAPI
RXRγNG2DAPI
O4RXRγ-NG2DAPI
NG2VDR-HDAC1DAPI

Fig. 3.8 Experimental set up for Duolink® assay. OPCs were isolated via mixed glia isolation and left for 48 hours in either presence or absence of GFs. OPCs were identified using the marker NG2 and split between cycling and non-cycling based on EdU incorporation. Newly formed oligodendrocytes were distinguished by the expression of the marker ENPP6 and the absence of MBP. Oligodendrocytes were identified using the mature oligodendrocyte marker MBP. The bottom set of immunocytochemistry pictures illustrate the biological controls used for the Duolink® assay. On the left the expression of RXRγ and NG2 is shown. The interaction between these two proteins was used as a negative control as no signal is observed when carrying out Duolink®. VDR-HDAC1 was used as a positive control as this interaction occurs throughout the whole lineage. Duolink® signal results in small, quantifiable, fluorescent puncta. Quantification was carried out by measuring the integrated intensity of the Duolink® signal in either the nucleus or cytoplasm of the cells and normalising it by the area over which the measurement was carried out. The signal was then normalised over the VDR-HDAC1 interaction at each stage.


A
Cycling OPC
Non-cycling OPC
Newly formed OLG
OLG
PLA Intensity/Area
1.5
1.0
0.5
0.0
****
VDR
RARβ
PPARγ
Nurr1
RARγ
PPARβ
LXRα
LXRβ
THRα
THRβ
NR interaction with RXRγ


B
RXRγ-LXRβ interactions
NG2EdUDAPI
NG2EdUDAPI
ENPP6MBPDAPI
ENPP6MBPDAPI

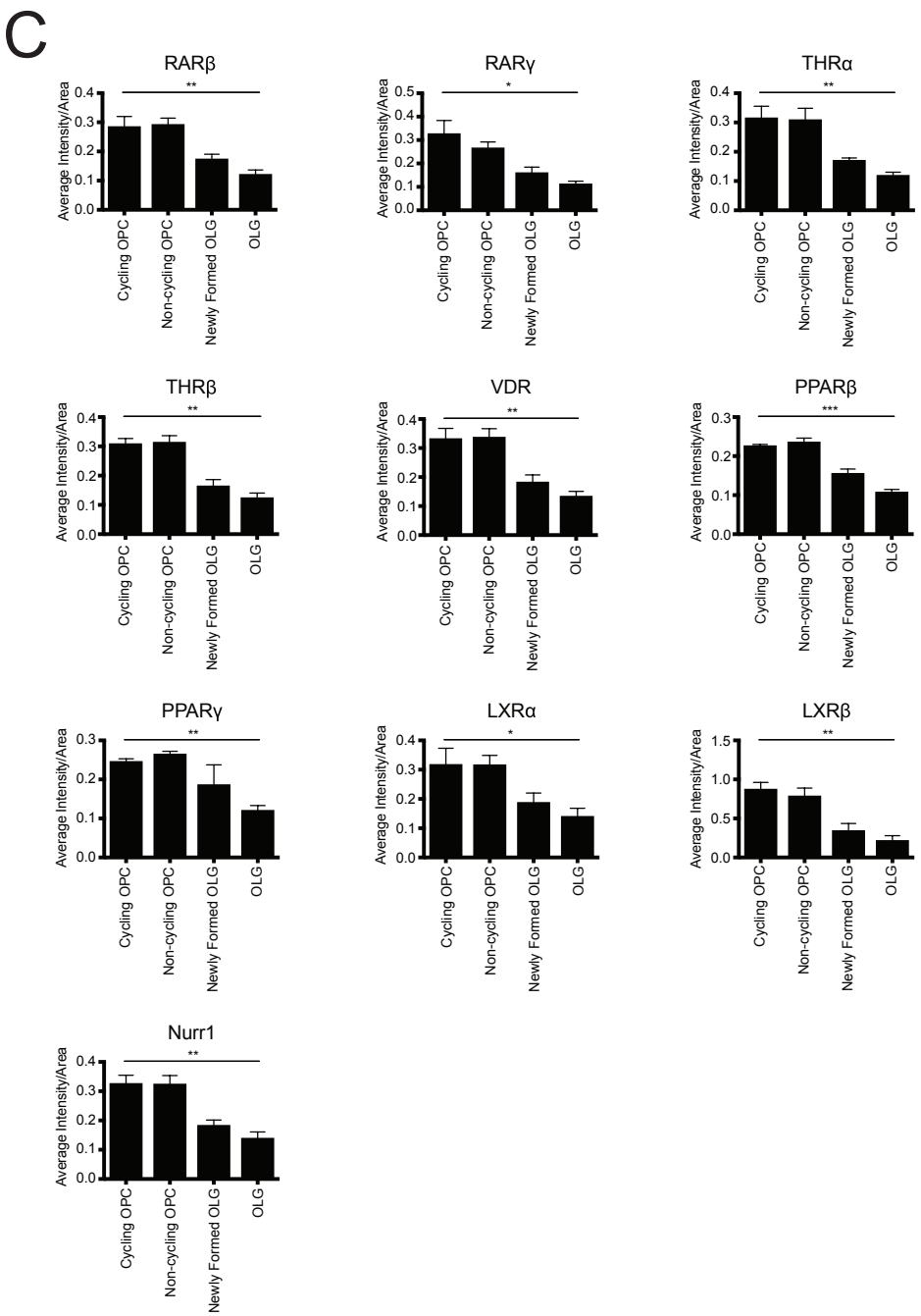


Fig. 3.9 RXRγ does not switch binding partners during OPC differentiation. Rat primary OPCs were isolated using mixed glia shake off and Duolink® was carried out for RXRγ and a variety of NR partners at different stages of the lineage. The nuclear Duolink® signal for each interaction was quantified. (A) There is no significant difference in the interaction of RXRγ to the array of binding partners assessed at the four different stages of the oligodendrocyte lineage. The only heterodimer showing a significant difference is RXRγ-LXRβ. This is true only at the initial stages of the lineage. (B) Immunocytochemistry of the RXRγ-LXRβ Duolink®. (C) When looking at how each RXRγ heterodimer changes over the course of OPC differentiation, all heterodimers present the same pattern. The highest interaction is at the two OPC stages of the lineage and as they differentiate the signal decreases significantly. All data was analysed using ordinary one-way ANOVA followed by Tukey's multiple comparison test where significance was reached, (*p≤0.05, **p≤0.01, ***p≤0.001, ****p<0.0001). All graphs are presented as mean ± SEM, biological n=3.

3.2.5 The subcellular location of RXR heterodimers during OPC differentiation

From the Duolink® data presented, it appears that RXRγ does not actively switch the partner it is bound to as OPCs differentiate into mature oligodendrocytes.

However, the relevance of different partners at various stages of the lineage may be determined at other levels of NR signalling control. One of these is subcellular location, a salient aspect of NR control as the confinement of the receptors to the cytoplasm prevents them from carrying out their genomic functions, whilst their transport to the nucleus allows them to elicit transcriptional control. To assess whether subcellular location of RXRγ-NR categorises heterodimers responsible for the progression of OPC differentiation, I carried out Duolink® analysis of RXRγ binding to the same set of NRs previously tested. However, this time the cytoplasmic signal was also measured, and a ratio between nuclear and cytoplasmic signal at the four stages of the lineage was quantified. This would aid in understanding how the overall binding of RXRγ and each partner assessed is distributed within each lineage stage, and whether the location of the heterodimers changes over time.

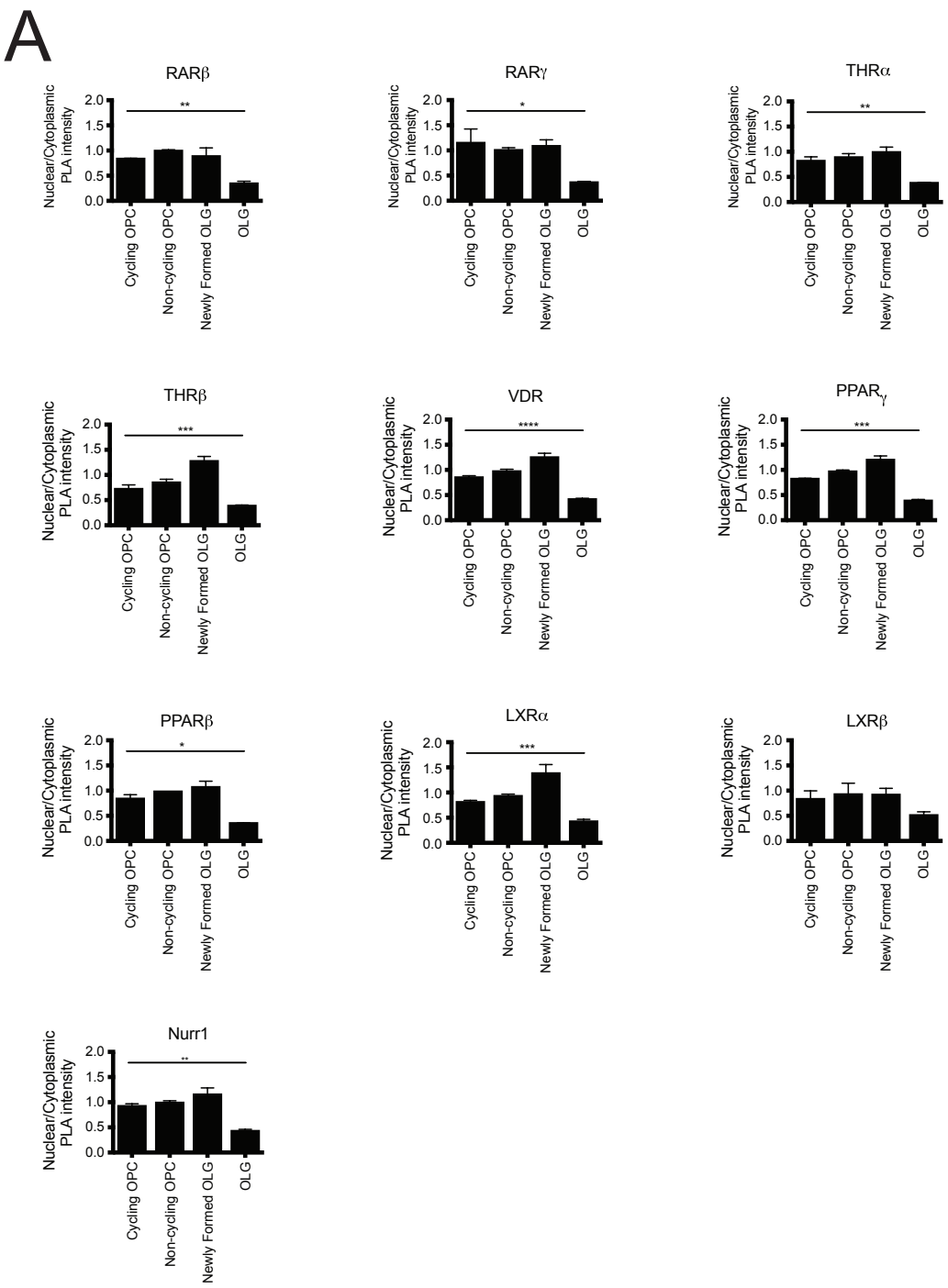
Figure 3.10A shows that all RXRγ-NR follow a similar pattern of distribution with regards to their nuclear:cytoplasmic ratio during OPC differentiation. Nuclear localisation of the heterodimers is highest at the initial stages of the lineage compared to cytoplasmic location. However, over the course of differentiation the ratio between nuclear:cytoplasmic signal location decreases (ordinary one-way ANOVA followed by Tukey's multiple comparison test where there is significance or Kruskall-Wallis test, n=3).

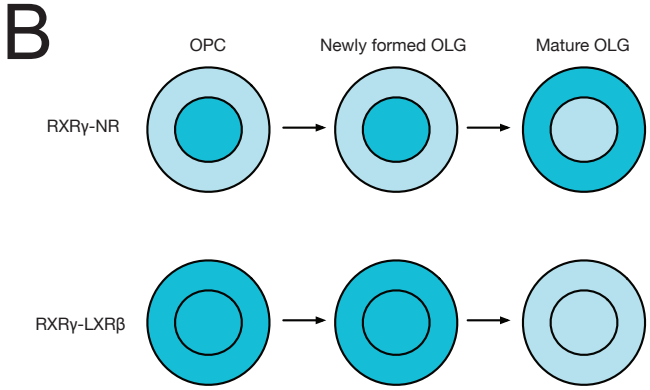
The lower ratio is due to the significant decrease in the nuclear signal as reported in Figure 3.9C of the previous section, but also due to an increase in cytoplasmic signal as differentiation occurs as shown in Figure 3.10C.

This is true for all heterodimers except for RXRγ-LXRβ which does not present a significant change in the ratio of nuclear:cytoplasmic location during OPC differentiation. This is because, unlike what is observed for other RXRγ-NRs, RXRγ-LXRβ levels also decrease in the cytoplasm as differentiation occurs, suggesting an overall reduction in the absolute amount of the heterodimer upon oligodendrocyte formation. The results are summarised by the diagram in Figure 3.10B.

The data presented shows a similar pattern of subcellular distribution for the majority of RXRγ-NR assessed throughout the oligodendrocyte lineage. At the initial stages of the lineage there is a high level of both nuclear and cytoplasmic heterodimers, which could be due to the high production of NRs necessary to be shuttled to the nucleus in order to induce differentiation as later discussed in Section 3.2.7. However, upon mature oligodendrocyte formation, the cytoplasmic signals increase compared to the previous newly formed oligo-

dendrocyte stage. This could be due to the heterodimers shuttling back to the cytoplasm once the necessary transcription regulation has occurred. Even though active shuttling of the receptors cannot be assessed with a static technique such as Duolink®, it has been reported that continuous nucleocytoplasmic shuttling is essential to the function of transcription factors such as Sox10, as upon inhibition of its nuclear export a reduction in the transactivation of both transfected reporters and endogenous target genes is observed (Rehberg et al., 2002). Similarly, prolonged localisation of GR in the nucleus of NIH3T3 cells due to reduced cytoplasmic shuttling resulted in increased luciferase expression compared to control when carrying out luciferase reporter assays (Black et al., 2001). This demonstrates that NR shuttling is important in controlling the expression level of target genes, and even though the observations presented were obtained via the use of artificial systems, it cannot be excluded that this could also occur at the level of endogenous gene expression in primary cells. The increase in cytoplasmic RXRγ-NR may therefore be due to shuttling-mediated control of gene transcription upon terminal differentiation. However, techniques such as FRET in combination with live imaging are better suited to determine whether active nucleocytoplasmic shuttling is indeed the reason for the cytoplasmic increase in RXRγ-NR in mature oligodendrocytes. On the other hand, the increase in cytoplasmic heterodimers could result from an increase in NR protein production and consequently RXRγ-NR formation, rather than shuttling itself. The reason for an increased requirement of RXRγ-NR at the final stage of the lineage is unclear, but it can be speculated that it may be for the non-genomic heterodimeric functions required for the appropriate maintenance of mature oligodendrocytes.


A
RARβ
RARγ
THRα
THRβ
VDR
PPARγ
PPARβ
LXRα
LXRβ
Nurr1
Nuclear/Cytoplasmic PLA intensity
2.0
1.5
1.0
0.5
0.0
**
*
***
****
Cycling OPC
Non-cycling OPC
Newly Formed OLG
OLG


B
OPC
Newly formed OLG
Mature OLG
RXRγ-NR
RXRγ-LXRβ

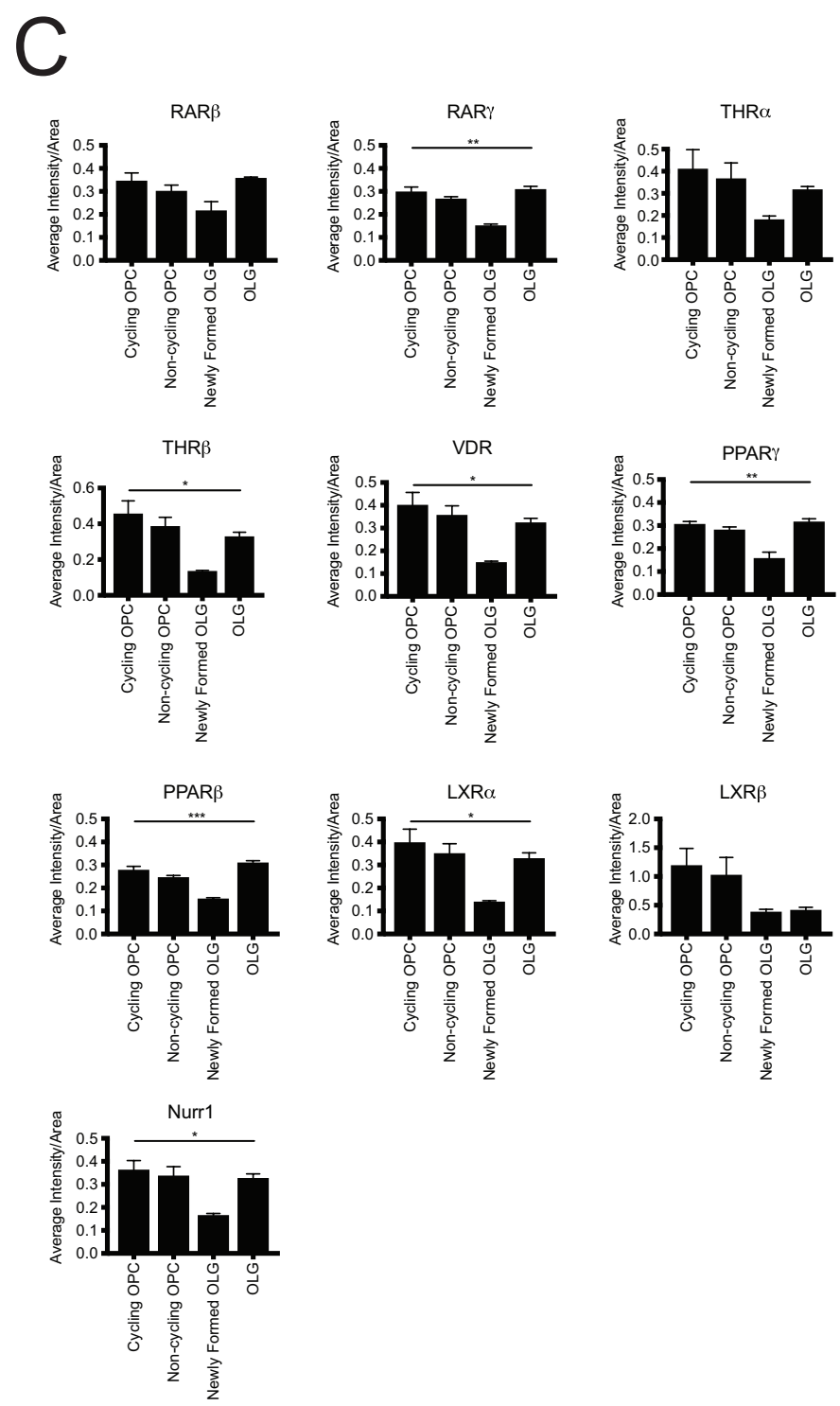


Fig. 3.10 Nucleocytoplasmic shuttling of RXRγ heterodimers over the course of OPC differentiation. Rat primary OPCs were isolated using mixed glia shake off and Duolink® was carried out for RXRγ and a variety of NR partners at different stages of the lineage. The nuclear and cytoplasmic signals were measured to determine changes in RXRγ-NR subcellular during differentiation. (A) The nucleus:cytoplasmic ratio of Duolink® signal changes significantly as OPCs differentiate. (B) Summary figure for heterodimer shuttling. The majority of heterodimers increase in their cytoplasmic presence and/or decrease in the nucleus as OPCs differentiate, except for RXRγ-LXRβ whose ratio remains constant. (C) Duolink® signal in the cytoplasm. All data was analysed using ordinary one-way ANOVA followed by Tukey's multiple comparison test where significance was reached (*p≤0.05, **p≤0.01, ***p≤0.001, ****p<0.0001). All graphs are presented as mean ± SEM, biological n=3.

3.2.6 Co-regulator association to RXR partners during OPC differentiation

The fourth level of NR signalling control consists of co-regulator association as shown in Figure 1.7. As little differences were observed between RXRγ-NR behaviours when assessing partner switching and changes in subcellular location throughout OPC differentiation, co-regulator association may be the major level of signalling control. Table 3.1 summarises an array of co-regulators that have been shown to influence both OPC and Schwann cell biology in some way. These have been broadly divided into either activators or repressors of gene expression. However, the same co-regulator can act as either depending on the overall machinery it associates to (Perissi and Rosenfeld, 2005).

To assess whether co-regulators switch dynamically in order to promote OPC differentiation, Duolink® analysis was carried out between each RXRγ partner previously assessed and the set of co-regulators reported in Table 3.1. The experiment was not carried out with RXRγ in order to achieve the specific co-regulator association to each individual heterodimer, thereby determining their activation in the different lineage stages.

As shown in Figure 3.11, throughout OPC differentiation co-regulators associate to RXR partners following one of two main patterns.

Co-regulator	Type	Effect	Reference
BRG1	Activator	Aids the expression of myelin genes	Marathe et al. (2013)
PGC1α	Activator	Increases myelination	Yoon et al. (2016)
SRC1	Activator	Affects P0 in Schwann cell lines	Cavarretta et al. (2004)
TRAP220	Activator	DRIP-complex components	Oda et al. (2010)
p300	Activator	Involved in OPC fate choice	Zhang et al. (2016a)
SIRT1	Repressor	Inhibits OPC differentiation	Rafalski et al. (2013)
SIRT2	Repressor	Inhibits OPC differentiation	Li et al. (2007)
HDAC1	Repressor	OPC differentiation	Ye et al. (2009)
HDAC2	Repressor	OPC differentiation	Ye et al. (2009)
HDAC3	Repressor	Involved in OPC fate choice	Zhang et al. (2016a)
HDAC11	Repressor	Inhibition impairs OLG maturation	Liu et al. (2009)
NCoR	Repressor	OPC fate choice	Castelo-Branco et al. (2014)

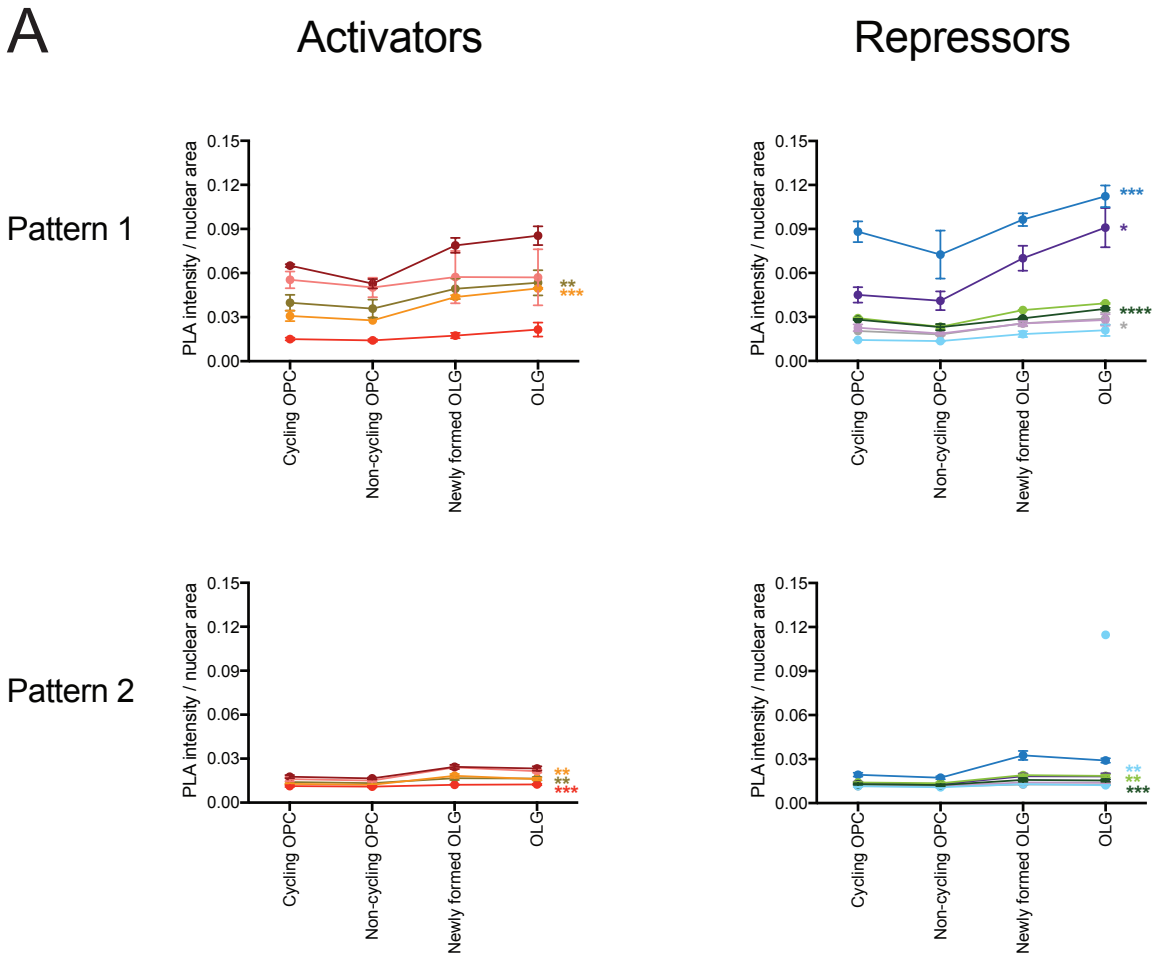
Table 3.1 Co-regulators involved in myelin cell biology.

LXRβ is shown as an example of pattern 1 of how co-regulator associate to the NRs being assessed, and PPARγ as an example of pattern 2. Pattern 1 association is observed for the majority of partners, where both activators and repressors increase in their association to the partner as differentiation occurs (ordinary two-way ANOVA followed by ordinary one-

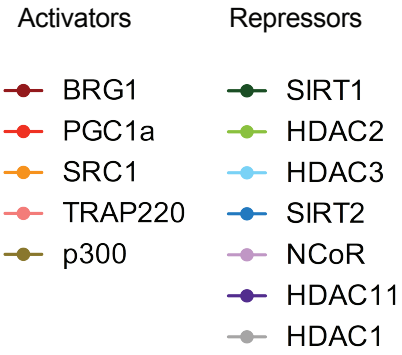
way ANOVA where there was significant interaction between the two variables assessed, n=3). The co-regulators reporting significant difference over the course of differentiation vary amongst different partners. On the other hand, pattern 2 reports a lower association of the same co-regulators to PPARγ and RARγ. Nevertheless, some report significant difference in their association over time depending on the partner they associate to.

Co-regulator association to RXRγ partners changes as OPC differentiation occurs and the specific regulators that present a significant change depend on the partner being assessed. Even though the exact biological relevance of these results remains too complex to interpret, it is clear that co-regulator binding to RXRγ partners is more dynamic than partner binding to RXRγ. Furthermore, co-regulator binding is dependent on the specific NR being assessed, suggesting that each RXRγ partner has their own inhibition and activation profile, which may be involved in the regulation of diverse cellular functions throughout the various differentiation stages.

A



B



C

Pattern	Followed by:
1	LXRβ
	LXRα
	THRα
	THRβ
	RARβ
	VDR
	Nurr1
2	PPARγ
	RARγ

Fig. 3.11 Co-regulator association to RXRγ partners is more dynamic. Rat primary OPCs were isolated using mixed glia shake off and Duolink® was carried out for the NR partners previously assessed and a variety of co-regulators at different stages of the lineage. The nuclear Duolink® signal for each interaction was quantified. (A) Co-regulators associate in one of two patterns. Pattern 1 shows the interaction of LXRβ with each co-regulator assessed, and pattern 2 shows the interactions of PPARγ with the same array of co-regulators. Which co-regulators result in significant changes depends on the partner being assessed. (B) The co-regulators picked and the equivalent colours used to represent them in the graphs in (A). (C) Table showing the co-regulator pattern presented by the partners assessed. The majority display pattern 1, whilst only PPARγ and RARγ present pattern 2. All data was analysed using ordinary two-way ANOVA. If interactions were observed, ordinary one-way ANOVA was carried out (*p≤0.05, **p≤0.01, ***p≤0.001, ****p<0.0001). All graphs are presented as mean ± SEM, biological n=3.

3.2.7 Discussion

In this section I have assessed the different levels at which NR signalling is controlled in primary neonatal OPCs. It is clear that NR ligands are salient components of the system, as the presence of single ligands do not affect OPC differentiation except in the case of THR activation via its ligand T3. Unlike what has been previously reported, RXR activation with 9cRA did not induce OPC differentiation in serum-free conditions, unless in combination with the presence of another NR ligand. These observations give confidence that the presence of multiple NR ligands is required for differentiation to occur, and that the effects observed by Huang et al. (2010a) occur via activation of an RXRγ heterodimer, rather than a monomer or homodimer.

On the other hand, the use of Duolink® to assess the partner switching hypothesis throughout lineage progression reported no bias in partner association to RXRγ, except for the RXRγ-LXRβ heterodimer. The data suggests that RXRγ is mainly associated with LXRβ at the early stages of the lineage, and this interaction falls to the same level of association exhibited by the other partners as OPCs differentiate. A link between LXR activation, increased myelin gene expression, myelination and remyelination has been previously described (Shackleford et al., 2013; Meffre et al., 2015), and these results point towards a fundamental role for RXRγ-LXRβ heterodimer signalling at the early stages of the lineage. An increase in cholesterol production and metabolism is essential to OPC differentiation as cholesterol is the major lipidic component of the oligodendrocyte plasma membrane and consequently myelin. The importance of LXRs in fatty acid and cholesterol metabolism has been already outlined in Section 1.6.3, and the high association between RXRγ and LXRβ at the OPC stage of the lineage could be essential for preparing the OPC to the necessary increase in cholesterol production, thereby aiding progenitors in meeting the metabolic demands required for successful differentiation.

A particularly interesting observation from this set of results is that all nuclear RXRγ-NR interactions are highest at the progenitor stage and decrease as differentiation progresses. It has been reported in the literature that stem cells present different chromatin structure and organisation compared to differentiated cells.

Indeed, both ESCs and iPSCs have diffuse and less compact chromatin as well as a high level of chromatin remodelling factors in their nucleus (Chalut et al., 2012; Mattout et al., 2011; Delgado-Olguin and Recillas-Targa, 2011). As cells differentiate the chromatin compacts and areas become transcriptionally inactive (Alcobia et al., 2000; Martou and De Boni, 2000; Alcobia et al., 2003; Santos et al., 2002). Chromatin remodelling factors such as NuRD are essential in ESC differentiation, pointing towards a role for chromatin remodelling factors in both maintenance of stem cell identity and in priming the cell for differentiation (Kaji

et al., 2006). These observations have lead to the hypothesis that as cells differentiate there is a need to induce specific expression programmes, and chromatin remodellers allow this to occur by forming chromatin domains. Epigenetic domains allow fast and easy access for transcription and regulatory factors that can silence genes involved in stem cell maintenance and promote a lineage-specific gene expression programme (Meshorer et al., 2006).

Being NRs key recruiters of chromatin remodelling machinery and regulatory factors (Egea et al., 2000; Aranda and Pascual, 2001; Evans and Mangelsdorf, 2014), the high levels of RXRγ-NR observed in OPCs reflect the need of progenitor cells to undergo chromatin domain specification in order to induce lineage-specification and differentiation. As progenitors differentiate, the requirement for RXRγ heterodimers decreases until differentiation is complete. Upon terminating the differentiation programme, mature oligodendrocytes enter a maintenance state where a low level of transcription is required and therefore a lower level of RXRγ-NR are present in the nucleus.

The majority of partners show a similar extent of RXRγ binding throughout the lineage stages assessed. Despite further validation using different techniques is required to confidently confirm the above, based on the Duolink® results it is tempting to conclude that the partner switching hypothesis is incorrect. However, although the partners may not physically switch their binding to RXRγ, the possibility of specific heterodimers being responsible for OPC lineage progression cannot be excluded. The extent of RXRγ binding may not necessarily imply biological function, and functionally predominant heterodimers may be determined at other control levels despite RXRγ being constantly bound to all of the partners assessed. Functional dominance could be obtained via ligand presence, as ligands control various aspects of NR signalling, including subcellular shuttling and co-regulator recruitment. Both of these were assessed. Changes in subcellular location are also similar amongst the different partners, however co-regulator recruitment and association is more dynamic and is dependent on both the cell's stage in the lineage and the partner being assessed.

This leads to an overall picture, summarised in Figure 3.12, where at each stage of the lineage RXRγ heterodimers are stable, and the biological relevance of the heterodimer is dependent on the dynamic association of co-regulators as differentiation progresses.

The co-regulator recruitment is probably determined by the ligands that the heterodimers are presented with, as it has been previously reported that co-regulator association is dependent on the conformational changes the heterodimers undergo upon ligand binding (Heery et al., 1997; Nolte et al., 1998; Gronemeyer et al., 2004).

Overall, the different control levels for NR signalling function in order to obtain a single outcome: regulation in the expression of target genes. This is because the ultimate physiological response is determined by the expression and repression of the desired set of genes.

It is therefore clear that in order to understand how RXRγ activation truly promotes OPC differentiation one needs to know the genes targeted by RXRγ in oligodendrocyte lineage cells.

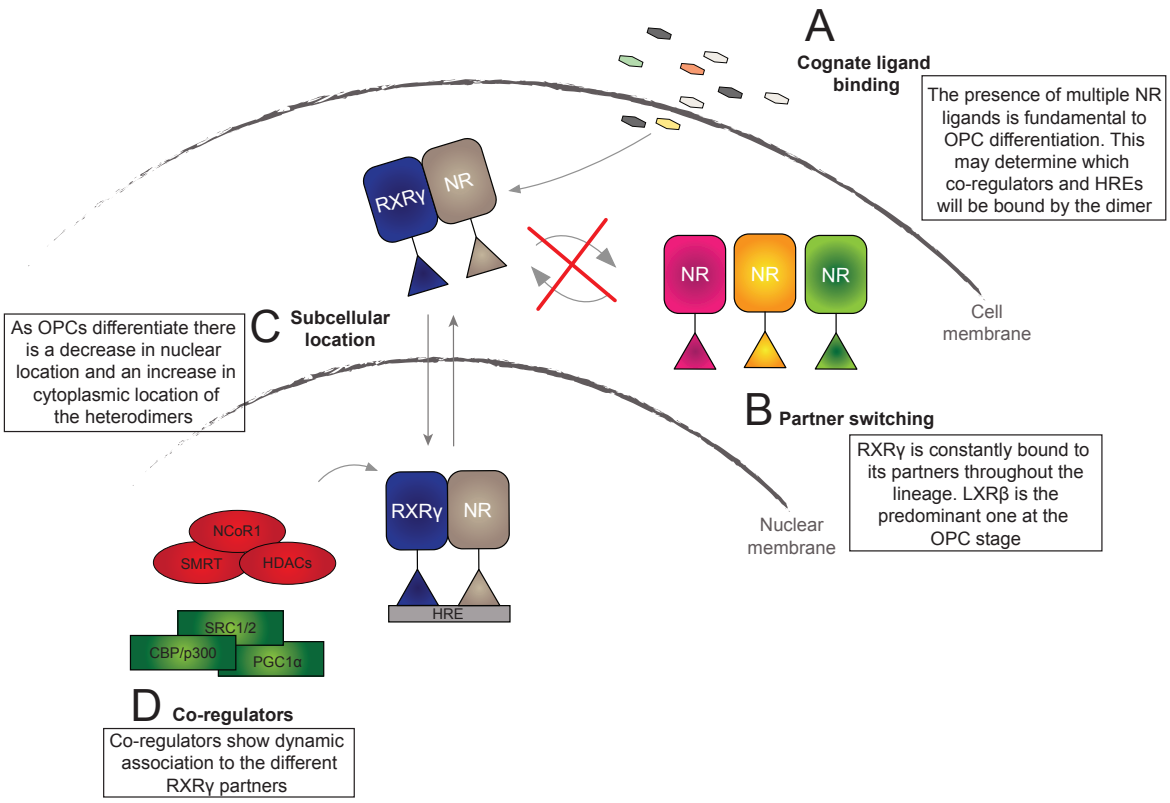


Fig. 3.12 Summary of the observations made on the levels of control for RXRγ-NR signalling in oligodendrocyte lineage cells. Multiple NR ligands need to be present in order to observe a differentiation effect via RXR activation. This is because in serum-free conditions and in charcoal-treated serum, 9cRA does not promote OPC differentiation. Based on the Duolink® assay results, RXRγ does not switch its binding partner at different stages of the lineage, but all partners are bound to the same extent. Only RXRγ-LXRβ show interaction prevalence at the OPC stage. There are significant changes in the nuclear and cytoplasmic locations of the heterodimers as OPC differentiation occurs, however it cannot be stated whether this is active heterodimer shuttling with the technique used. Co-regulator association to the RXRγ partners is very dynamic, dependent on the partner being observed and likely regulates the expression of different sets of genes that are key to different cellular functions.

3.3 Identifying the genes controlled by RXR in oligodendrocyte lineage cells: a ChIP-seq approach

In the last section I have assessed how RXRγ signals and the levels at which the signalling network are controlled. However, the ultimate goal of this study is to understand how RXRγ activation translates into OPC differentiation. In Section 3.1 I have described how NRs have non-genomic roles as well as genomic ones (Boonyaratanakornkit et al., 2001; Skildum et al., 2005; Hammes and Levin, 2007; Sarkar et al., 2008; Ordóñez-Morán et al., 2008). Therefore, it cannot be excluded that OPC differentiation could be elicited via a non-genomic RXRγ pathway. However, NRs have been for the most part described and studied due to their role as transcription factors. As the process of differentiation requires profound changes at the transcriptional level, I hypothesised that RXRγ activation leads to the transcriptional regulation of genes specifically involved in OPC differentiation. THR, LXR and VDR have all been shown to influence the expression levels of genes important to oligodendrocyte formation, such as MBP and PLP, and HREs for some of these receptors have been identified in the promoter regions of these genes (Farsetti et al., 1991; Meffre et al., 2015; de la Fuente et al., 2015; Shackleford et al., 2017). However, it is not currently known whether the transcriptional control of myelin-specific genes by these receptors occurs via RXR heterodimers, as the genes controlled by RXRγ in OPCs have not been assessed to date. In order to tackle this question, I decided to carry out ChIP-seq for RXRγ in primary rat OPCs.

ChIP-seq is used to map genome-wide binding sites of transcription factors and histones using chromatin immunoprecipitation (ChIP), in combination with DNA sequencing. The sequenced DNA fragments are then mapped back to the genome, in order to determine the DNA sites at which the protein of interest binds. Figure 3.13A illustrates the ChIP-seq experimental design. I used MACS sorted neonatal rat OPCs for ChIP-seq, preventing any exposure to serum, as the presence of other NR ligands could alter RXRγ binding. The progenitors were then plated into T75 flasks and left to recover and proliferate in the presence of GFs for five days until confluent. They were then treated for 24 hours with 50nM 9cRA or 0.1% DMSO as vehicle control. The two treatments would allow the assessment of RXRγ association to the genome under both basal and activated conditions. ChIP was carried out using both an anti-RXRγ antibody and a nIgG control in order to assess non-specific background binding in both conditions. A biological replicate of three was used for all the samples and controls.

Despite the continuous evolution of the technique, ChIP-seq still presents some challenges with regards to the experimental design, especially when using primary cells. One of these limitations is the cell number required to carry out the experiment. ChIP-seq for

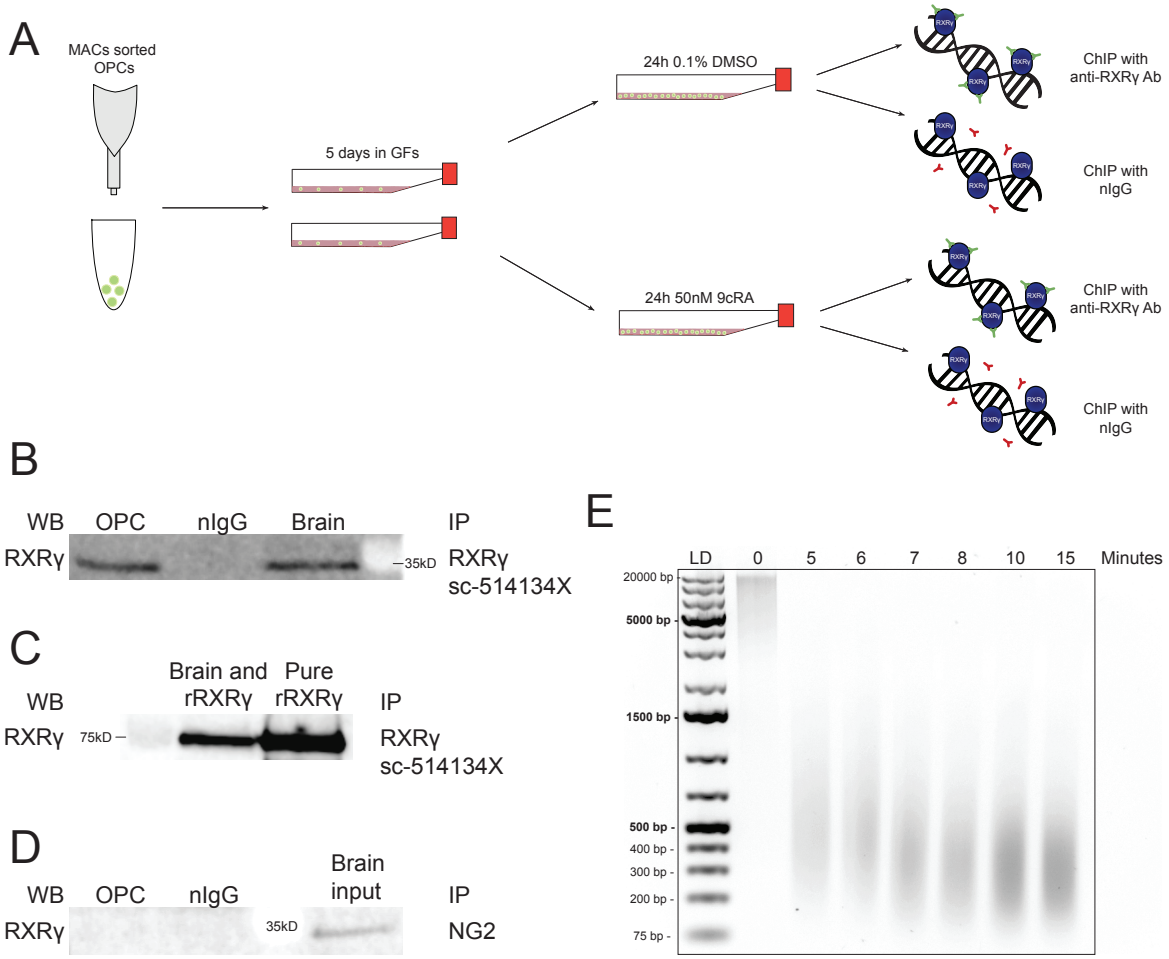


Fig. 3.13 Experimental design and controls for RXRγ ChIP-seq in primary rat OPCs. (A) Illustration of the ChIP-seq experimental design. OPCs were isolated via MACS and plated in T75 flasks. They were kept for five days in GFs until confluent. The OPCs were then treated for 24 hours with either 50nM 9cRA or 0.1% DMSO as vehicle control. The OPCs were detached and ChIP using either an anti-RXRγ antibody or a nIgG control was carried out. (B) Western blot showing the CoIP binding of the chosen RXRγ antibody (sc-514134X). RXRγ antibody ab15518 was used for the detection. The nIgG control shows no RXRγ pulldown. (C) Blot showing that RXRγ sc-514134X antibody binds recombinant RXRγ. (D) Blot showing a further control where CoIP was carried out using anti-NG2 antibody. When RXRγ is detected, no band is revealed. (E) 1% agarose DNA gel of the chromatin shearing trial. The length of the shearing trials is shown at the top of the gel. The majority of the fragments need to fall within 400-200bp fragment size, therefore an overall shearing time of 6 minutes was selected.

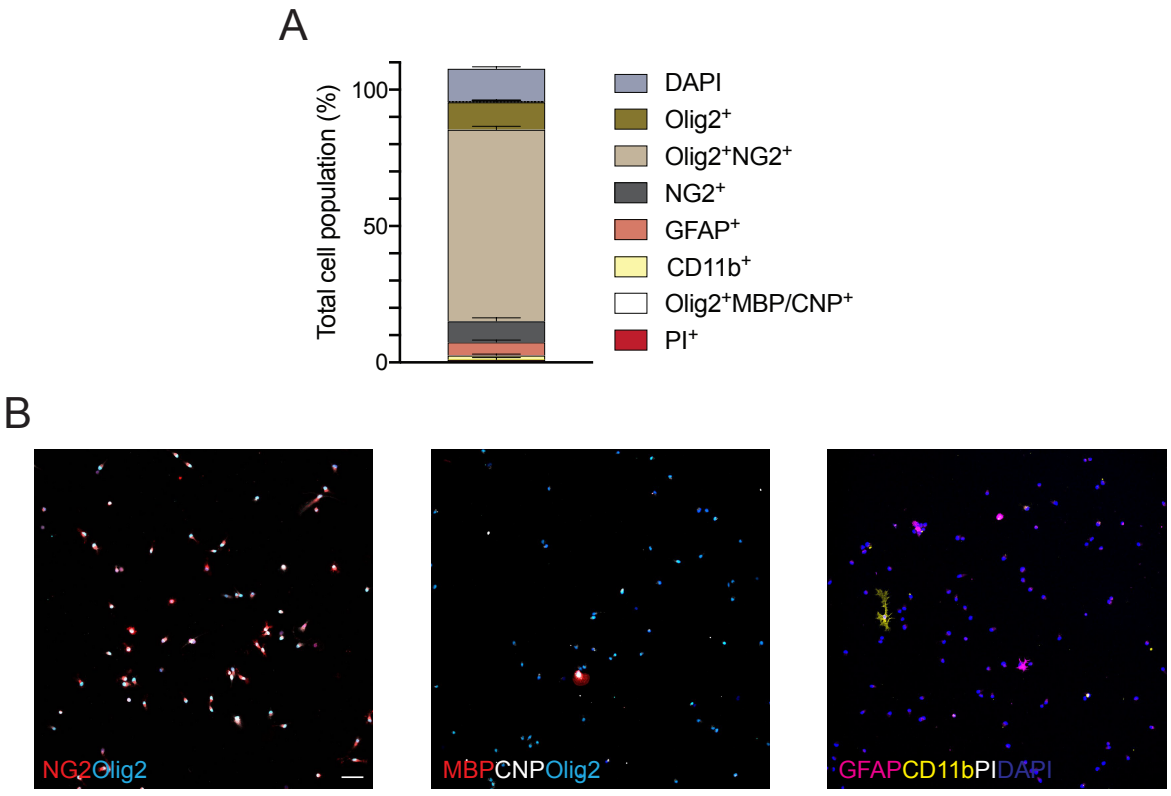


Fig. 3.14 Purity of OPC cultures prior to ChIP-seq. Primary rat OPCs were isolated using MACS and kept for five days in culture in the presence of GFs in order for the progenitors to proliferate. After five days the cells were detached, plated on coverslips and left to recover for 2 hours before fixing and assessing their purity via immunocytochemistry. (A) The number of $Olig2^+NG2^+$ nuclei is on average 70.25% ± 2.001%. Astrocytic and microglial contamination was minimal, with 4.85% ± 1.278% of $GFAP^+$ and 1.57% ± 0.783% of $CD11b^+$ nuclei. 10.04% ± 0.594% of nuclei expressed only Olig2; however, no mature oligodendrocytes are present, with (0.23% ± 0.398% of either $CNPase^+$ and MBP^+ cells). 7.66% ± 2.021% of the nuclei were positive only for the marker NG2, and almost no cell death was observed in the cultures, as the percentage of PI^+ nuclei was 1.09% ± 1.173%. Overall, this data shows that the cultures obtained form the experimental set up for ChIP-seq result in a relatively pure culture highly enriched for OPCs with minimal astrocyte formation and proliferation of other undesired cell types. (B) Representative immunocytochemistry pictures for graph (A). All graphs are presented as mean ± SEM, biological n=3, scale bar = 50μm.

transcription factors requires 10-20 million cells/sample (Park, 2009), which would require the use of an excessive number of animals when using primary rat OPCs. It is for this reason that I allowed the OPCs to proliferate for 5 days *in vitro* and carried out an initial sequencing trial using 5 million OPCs/sample. This yielded enough DNA for genes to be detected. Thus, 5 million OPCs/sample were used in this set of experiments. Secondly, the experimental outcome of ChIP-seq is highly dependent on the quality of the primary antibody used (Park, 2009). For this experiment I picked the mouse monoclonal anti-RXRγ antibody from Santa Cruz Biotechnology (sc-514134X). My decision was based on the literature, as ChIP-grade RXR antibodies from Santa Cruz have been used in previous ChIP experiments (Daniel et al., 2014). Further CoIP validation followed by western blot of anti-RXRγ sc-514134X specifically, revealed the single 35kD band of RXRγ2. This same band does not appear when using the mouse nIgG for CoIP.

A source of artefacts in the experimental outcome could arise from the chromatin shearing step of the ChIP protocol (Section 2.2.14). The optimal size of the DNA fragments prior to ChIP should be between 200-400bp and the uniformity of the chromatin shearing step needs to be constant between the different samples. To determine the optimal shearing conditions I have carried out shearing trials by changing the number of cycles whilst keeping the sonicator power, cycle lengths and concentration of OPCs/sample constant. One of the DNA gels ran is shown in Figure 3.13E. The shearing process should aim at obtaining the majority of the chromatin sheared to the required fragment size with a minimal number of cycles. This is because over-shearing can result in detachment of the cross-linked proteins from the DNA. Based on the trials carried out I picked 6 shearing cycles. Upon completion of the control experiments, the optimal conditions for the ChIP-seq were determined and the experiment was carried out.

In Section 3.2.2 I have shown that OPCs differentiate into type II astrocytes *in vitro* when the plating density is too high. To ensure that five days in proliferative conditions did not cause astrocyte formation, OPCs were detached and re-plated on coverslips, left to recover for 2 hours and stained in order to assess the purity of the culture. Figure 3.14 shows that an average of 4.85% (SD=1.278 , n=3) of the DAPIs were positive for the astrocytic marker GFAP. Contamination by microglia and NG2-only cells was minimal, with an average of 1.57% (SD=0.78, n=3) CD11b$^+$ cells and 7.66% (SD=2.02, n=3) NG2$^+$ cells. With regards to the oligodendrocyte lineage there were almost no mature oligodendrocytes present (on average 0.23%, SD=0.40, n=3), the percentage of Olig2-only cells was on average 10.04% (SD=0.59, n=3), whilst the number of OPCs made up on average 70.25% (SD=2.00, n=3) of the total cell population. The cultures presented almost no cell death as the average of PI$^+$ cells was 1.09% (SD=1.17, n=3) and there were very few unidentified cells which stained

for none of the markers assessed (on average 12.05%, SD=0.70, n=3). Overall, this suggests that the cultures obtained for ChIP-seq are enriched in OPCs with minor contamination from other cell types.

3.3.1 Genome-wide profiling of RXR in primary rat OPCs

I used ChIP-seq to define RXRγ occupancy after 24 hours of either 0.1% DMSO (vehicle control) or 50nM 9cRA treated OPCs. This would aid in assessing the basal occupancy of both inactive and active RXRγ. The peaks obtained from the sequencing were aligned to the latest rat genome (rn6) and called using model basis analysis of ChIP-seq 2. The peaks were compared to the ones obtained via ChIP of the nIgG control to remove any unspecific background antibody binding. Once the background binding was removed, the peaks with a q value lower than 0.5 detected in two or more biological replicates were considered to be areas of RXRγ binding. This data processing was kindly carried out by Dr. Sabine Dietmann at the Wellcome Trust Stem Cell Institute at University of Cambridge.

It is conventionally accepted that, unlike class III NRs, RXR heterodimers do not always shuttle to the nucleus upon activation. Instead, they can be found already associated to the genome in the absence of their cognate ligands, and carry out the desired effect upon ligand binding. I thus assessed the genome-wide occupancy of RXRγ at both basal conditions as well as after 9cRA exposure (Figure 3.15A). Few peaks were observed in vehicle treated primary OPCs, revealing that the basal occupancy of RXRγ is minimal in the absence of its ligand. The Venn diagram in Figure 3.15B shows that approximately 111 peaks were called in DMSO treated OPCs, a small number compared to the 12,372 peaks called in 9cRA treated OPCs. The two conditions only presented 11 peaks in common. Multiple EM for Motif Elicitation (MEME) analysis was used for the discovery of *de novo* sequence motifs bound by RXRγ in the ChIP-seq carried out. This analysis revealed that RXRγ consistently binds to the motif shown in Figure 3.15C, which highly resembles the previously reported core motif for NRs AGGTCA, as over 50% of the bases are in common between the two motifs. The low variability of the RXRγ binding motif here reported gives further confidence that the peaks derived from the ChIP-seq are not coincidental and that the data obtained can be further analysed.

NRs act as transcription factors and it has been reported that they mainly exert their function by binding to promoter regions in close proximity of the transcription start site (TSS) of the gene of interest (Umesono et al., 1988; Evans and Mangelsdorf, 2014). Figure 3.15D illustrates the distribution of the peaks around their closest TSS. In contrast to what has previously been suggested, this data shows that the majority of the peaks reside up to 15kb upstream or downstream of their closest TSS. Therefore, the RXRγ binding sites

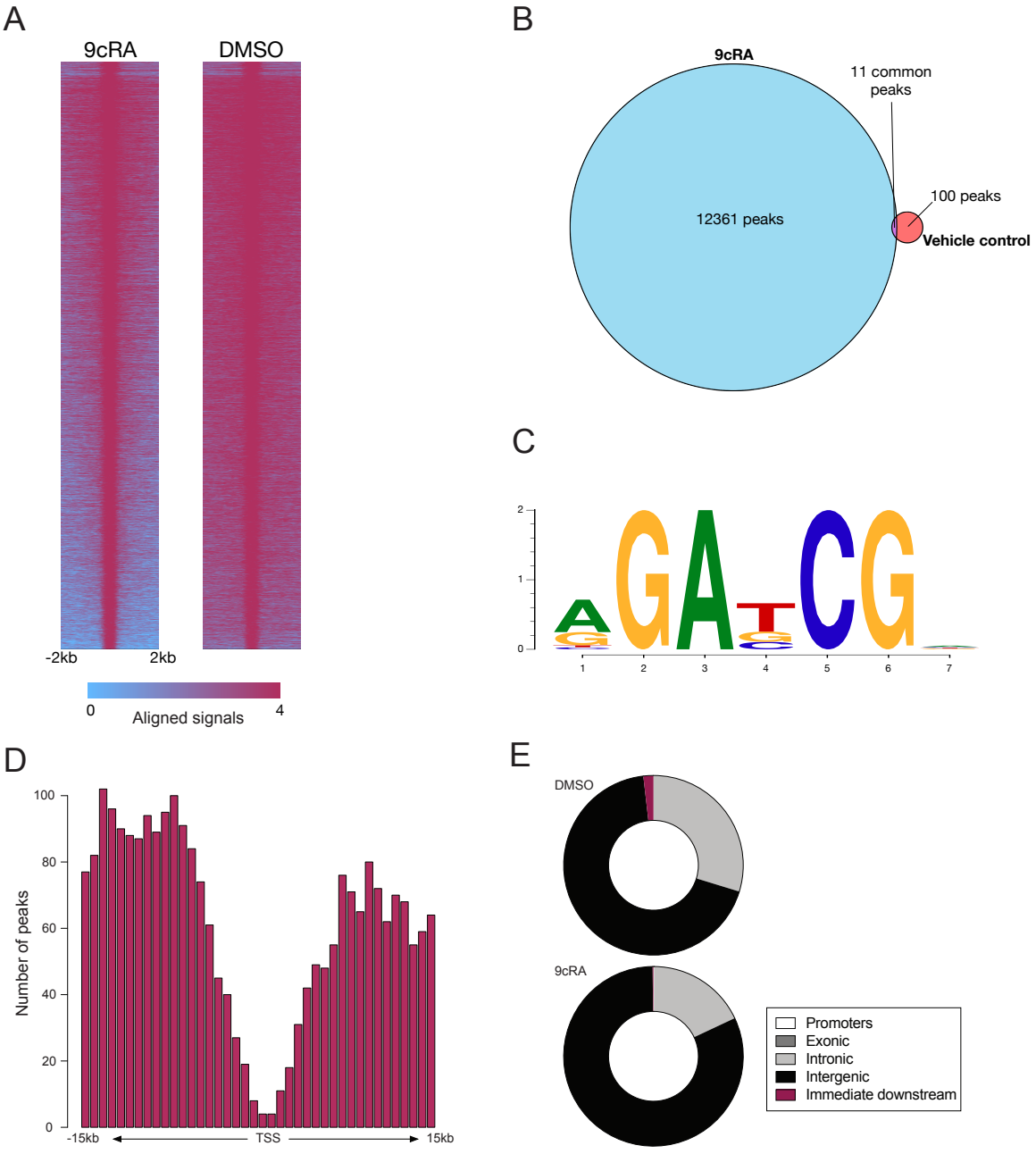
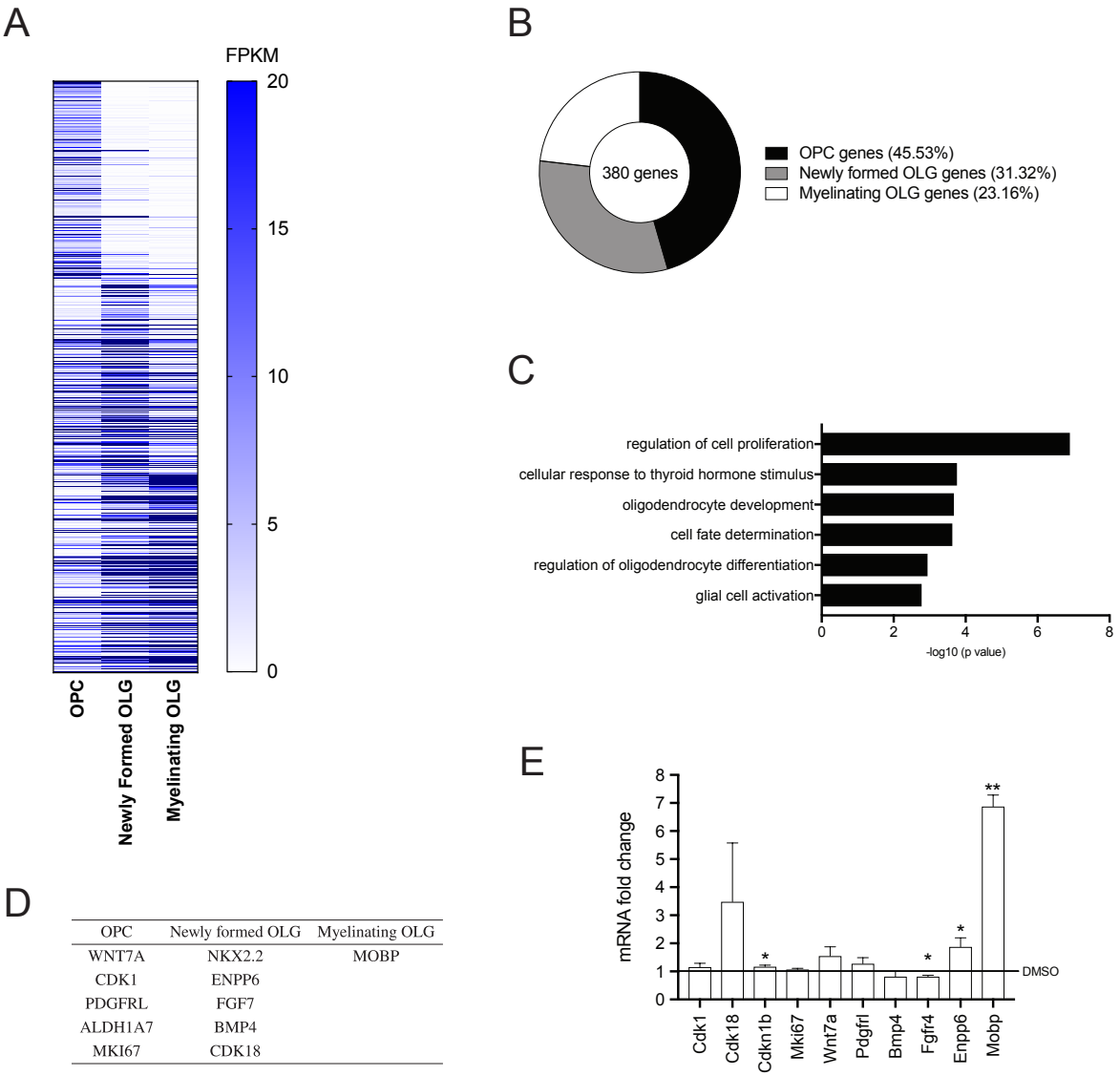


Fig. 3.15 Genome-wide RXRγ binding in the absence and presence of 9cRA. (A) Heatmaps of RXRγ-binding signals in rat primary OPCs after 9cRA exposure (left) and control conditions (right). Each line on the y axis represents a genomic region $\pm$ 2kb flanking RXRγ summits. This reveals that there is a higher number of peaks after 9cRA exposure compared to control. This is further illustrated by the venn diagram (B) showing that the global RXRγ occupancy under control conditions, with 111 total peaks called. 9cRA exposure resulted in 12372 RXRγ peaks, suggesting that the presence of an agonist promotes genomic-binding of RXRγ. (C) MEME analysis revealed that RXRγ binds to a constant motif throughout the genome. This motif closely resembles the previously reported core motif for NRs AGGTCA. (D) Analysis of the distribution of peaks around the closest TSS in 9cRA conditions shows that the majority of peaks are located far away from TSS, up to 15kb upstream or downstream of it. (E) Genomic annotations of the peaks shows that the majority of the RXRγ peaks are found in intergenic and intronic genomic areas, and almost none are found in promoter regions where NRs have been reported to act.

are relatively far away from promoter regions, here considered to be 5,000bp up and downstream of the TSS. Analysis of the genomic location of these peaks (Figure 3.15E) shows that RXRγ rarely associates to promoter regions in both control and 9cRA-treated conditions, with almost no promoters bound in both cases. Instead, it is mainly bound to intronic and intergenic genomic regions. At basal level, 29.73% and 68.47% of the RXRγ peaks fall within intronic and intergenic regions respectively, and upon 9cRA activation the peaks redistribute to 17.82% and 81.84%. ChIP-seq experiments on other NRs reveal the same pattern of association, regardless of whether they have been carried out on cell lines or primary cells (Carroll et al., 2005; Ramagopalan et al., 2010; Boergesen et al., 2012; Singh et al., 2015). These genomic areas make up the majority of the genome and are known to contain numerous regulatory elements for gene transcription, including silencers and enhancers, able to determine the likelihood of gene transcription to be promoted or prevented (Kolovos et al., 2012). The elements contained in these areas and their exact mechanism of function are still not completely understood, and the implication of RXRγ potentially binding to regulatory elements in OPCs will be further discussed in Section 3.3.3.

3.3.2 RXR HREs are located in proximity of genes involved in regulation of proliferation and stem cell control

When OPCs are exposed to 9cRA, RXRγ binds to numerous genomic areas resulting in 12,372 peaks. Despite this does not directly translate to 12,372 genes bound, it still reveals that RXRγ could bind to almost half of the rat genome, an observation which may not be surprising considering the plethora of cellular functions that RXR and its heterodimers influence. Gene ontology (GO) analysis of all the peaks obtained (data not shown) results in a large variety of cellular and biological processes, making it hard to identify which of these may be specific to oligodendrocyte differentiation. To refine and simplify the search, I used the database generated by Zhang et al. (2014) to aid in the process. Using this database I created a unique gene signature list of the top 500 differentially expressed genes between the three stages of the oligodendrocyte lineage that Zhang et al. (2014) analysed. This is illustrated by the heat map in Figure 3.16A. The top 500 genes for OPCs are expressed at extremely low levels in newly formed and mature oligodendrocytes. However, the expression levels of the unique gene signatures observed by the other two stages are less clear cut. This is because the newly formed and mature oligodendrocyte are effectively the same cell transitioning through different stages of the lineage, rather than two different cell types. Therefore, it is not surprising that the difference in gene expression between the two is not as sharply defined as the one with OPCs.



D

OPC	Newly formed OLG	Myelinating OLG
WNT7A	NKX2.2	MOBP
CDK1	ENPP6	
PDGFRL	FGF7	
ALDH1A7	BMP4	
MKI67	CDK18	

Fig. 3.16 RXRγ mainly associates to genes unique to OPCs and newly formed oligodendrocytes. (A) Heat map of the top 500 differentially expressed genes for OPCs, newly formed and myelinating oligodendrocytes according to Zhang et al. (2014). (B) I assessed whether RXRγ binds to any of these unique signatures, which it does to 380 of them. (C) GO term analysis of the 380 genes revealed that RXRγ mainly associates in proximity of genes involved in cell cycle regulation, proliferation, oligodendrocyte development and differentiation. (D) Table of the most interesting genes I have found RXRγ to associate to, based on the results of the GO term analysis and filtered based on the literature. (E) Graph showing the validation of the ChIP-seq using qPCR. qPCR was carried out on MACS isolated OPCs after 24 hours of DMSO or 9CRA exposure. All results were normalised to DMSO, shown in the graph to always be at a baseline value of 1. The fold change in the expression of each gene target assessed is shown. The expression of *Cdkn1b* (the transcript for p27), *Enpp6* and *Mobp* increase significantly after 24 hours of 9cRA exposure, whilst the expression of *Fgfr4* decreases significantly compared to control conditions. Data was analysed using Unpaired Student t Test, n=3 (*p≤0.05, **p≤0.01). Graphs are presented as fold change ± SEM, biological n=3.

The unique gene signatures were compared against the peaks obtained from the ChIP-seq, resulting in a total of 380 unique genes having RXRγ bound either in their proximity, promoter or intronic regions (Figure 3.16B). Division of the 380 genes between the three lineage stages revealed that 45.53% of the genes are unique to OPCs, 31.32% are unique to newly formed oligodendrocytes and 23.16% are unique to mature oligodendrocytes. This is an interesting observation as the majority of genes do not belong to the mature oligodendrocyte stage, as initially hypothesised. Instead, the majority of genes belong to OPCs and newly formed oligodendrocytes. GO term analysis of the 380 unique genes was carried out using the online software Gene Ontology Consortium (http://geneontology.org). Figure 3.16C reveals that RXRγ binding sites occur in proximity of genes involved in regulation of TH signalling, oligodendrocyte differentiation and development, as well as cell proliferation and stem cell biology.

To validate some of the targets of interest summarised in Figure 3.16D, I carried out qPCR for these genes. OPCs were isolated via MACS and treated for 24 hours with either 0.1% DMSO or 50nM 9cRA as with the ChIP-seq experimental conditions. They were then lysed and qPCR was carried out. The graph in Figure 3.16E summarises the results obtained. The expression level of the genes assessed upon 9cRA treatment was normalised against their basal expression level in the vehicle control sample. For this reason, the graph represents the fold change in the mRNA levels of the genes being assessed upon RXRγ activation (Unpaired Student t Test, n=3). Four of the genes change in their expression level upon 9cRA treatment; these are *Cdkn1b*, *Fgfr4*, *Enpp6* and *MOBP*. This suggests that RXRγ affects their expression, giving further confidence in the list of genes obtained by the ChIP-seq experiment.

3.3.3 Discussion

In this section I set out to find which genes are controlled by RXRγ in primary rat OPCs. I initially hypothesised that OPC differentiation as a consequence of RXRγ activation occurs because RXRγ heterodimers control the transcription of genes involved in mature oligodendrocyte formation. I therefore carried out ChIP-seq for RXRγ in neonatal rat primary OPCs treated with either 0.1% DMSO or 50nM 9cRA. As suggested in the literature for other NRs, genome-wide profiling of RXRγ revealed that the receptor mainly associates to intergenic and intronic DNA regions instead of genomic promoter regions (Carroll et al., 2005; Ramagopalan et al., 2010; Boergesen et al., 2012; Singh et al., 2015).

It is known that eukaryotic genomes are predominantly composed of non-protein coding DNA found between protein coding genes, as well as protein coding exons. In the 50s and 60s it was suggested that these genomic areas could activate and regulate transcription at certain

developmental and cell-specific differentiation stages (McClintock, 1950; Britten and Davidson, 1969), but despite these observations, for many years the general consensus remained that these areas were evolutionarily redundant. Instead, there is increasing evidence that non-protein coding regions are of great evolutionary importance, as they have been described to have functional relevance in the control of gene expression in both a condition-dependent and tissue-specific manner. Indeed, the analysis of sequenced genomes revealed that relative amount of non-protein coding DNA increases alongside the increasing complexity of an organism (Taft et al., 2007), with biological complexity being defined as the number of different cell types within an organism and their organisation (Vogel and Chothia, 2006; Taft et al., 2007). On the contrary, the amount of protein coding genes remains relatively static across different metazoa. Furthermore, the use of RNA-seq datasets from a variety of different animals has shown that the majority of non-protein coding DNA is transcribed in a tissue-specific manner and these areas may be important in the development and correct functioning of complex organisms (Liu et al., 2013).

The almost exclusive association of RXRγ to non-protein coding DNA regions in OPCs initially came as a surprise, as the role of NRs have largely been described in the promoter regions of protein coding genes. However, after observing ChIP-seq data of other NRs, such as VDR and GR, in a variety of cell types, it is clear that all NRs predominantly associate to intergenic and intronic genomic regions (Carroll et al., 2005; Ramagopalan et al., 2010; Boergesen et al., 2012; Singh et al., 2015). Non-coding DNA regions contain regulatory elements fundamental to the control of gene transcription, including transposable elements, alternative upstream promoters, enhancers and silencers, able to produce non-protein-coding RNAs with the capability of influencing the transcription of protein-coding genes kilobases away (Liu et al., 2013; Li et al., 2016). Regulatory elements have been shown to play fundamental roles in the correct outcome of biological processes such as development and regeneration. Single cell RNA-seq revealed that the expression of four ultra-conserved enhancers on the X-chromosome correlates with the expression of the *ARX* gene in different cell types in the brain (Dickel et al., 2018). *ARX* is a gene fundamental to neurological development. Dickel et al. (2018) used transgenic reporter mice lacking individual or a combination of these enhancers and followed their development. They observed that all animals were viable, but presented brain defects as a consequence of altered neuronal populations and brain structure, due to impaired *ARX* expression during development in the different cell types. In this study, Dickel et al. (2018) clearly demonstrated the functional importance of highly conserved non-coding genomic areas in driving biological processes fundamental to life. Enhancer elements have also been involved in regenerative processes. RNA-seq revealed an increased expression of *lepb* in regenerating zebrafish fins and heart compared to con-

trol tissue (Kang et al., 2016). Two enhancer elements in proximity of *lepb* were identified via the open chromatin mark Histone H3 acetyl Lys27 (H3K27ac) during tissue regeneration, but not in uninjured tissue. With the use of enhancer-effector transgenes using *lepb*-linked sequences upstream of factors known to influence regeneration, Kang et al. (2016) showed that these enhancers can indeed influence regeneration efficiency, suggesting their great potential for the development of regenerative therapies. Another interesting consideration comes from the recent sequencing of the axolotl genome (Nowoshilow et al., 2018). Among vertebrates, axolotls have no equal in their capability of adult regeneration and the recent sequencing of their genome will open new avenues in the study of regeneration, giving great hope to regenerative medicine. Interestingly, axolotls have one of the biggest genomes ever to be sequenced, comprised of 32Gb, ten times that of the human genome (Nowoshilow et al., 2018). One of the reasons for this is the huge expansion of intronic and intergenic non-protein coding regions (Nowoshilow et al., 2018). Whilst further studies are required to determine the reason for such an expansion, based on the papers previously mentioned it is tempting to speculate that these areas may be packed with regulatory elements which allow for the remarkable regenerative capacity of this animal model.

With regards to oligodendrocyte biology, the lineage-specific transcription factor Olig2 has been shown to recruit chromatin remodellers to enhancer elements involved in oligodendrocyte differentiation (Yu et al., 2013). Functional studies of Olig2 patterning revealed epigenetic marks such as H3K27ac around stage-specific enhancer elements that control the expression of salient regulators of OPC differentiation. These include MRF, Sox10, Olig1, Olig2 and Zfp191 (Yu et al., 2013). Regulatory elements found in intronic and intergenic regions are therefore important in numerous cell and tissue-specific functions.

Based on the ChIP-seq data I have here reported, RXRγ binds to areas abundant in regulatory elements able to influence gene transcription, raising two fundamental points:

1. RXRγ does not directly influence gene transcription, but instead may determine the likelihood and probability of a gene of interest being transcribed;

2. the proximity of an RXRγ binding site to a specific gene does not necessarily mean that that gene is the one being influenced by RXRγ.

In order to address the first point, ATAC-seq (assay for transposase-accessible chromatin using sequencing) alongside either RNA-seq or ChIP-seq for RNA polymerase could be carried out in 9cRA treated primary OPCs. The first technique would aid in understanding which chromatin areas are made available for transcription by RXRγ, while the second would give further confidence of which genomic areas are actively being transcribed as a consequence. In order to distinguish enhancer elements bound by RXRγ, ChIP-seq for H3K27ac would

need to be carried out and compared to the RXRγ here collected. The Richard Lu laboratory will kindly give us access to the OPC rat enhancer database they have developed based on H3K27ac distribution across the genome of developing OPCs. Despite the data was not collected for OPCs exposed to 9cRA, it will be an interesting starting point to map potential enhancer regions bound by RXRγ.

In order to map which regulatory elements influence which genes, functional studies need to be carried out. However, there is value in assuming that intergenic regions influence the genes closest to them and intronic regulatory elements influence the expression of the genes they are located in, as this is widely accepted in the field. The GO term analysis shown in Figure 3.16C suggests that the majority of genes specific to the oligodendrocyte lineage influenced by RXRγ have to do with cell proliferation and aspects of cell cycle regulation, rather than with oligodendrocyte maturation and myelination. The subsequent qPCR validations have shown that ENPP6 and myelin-associated oligodendrocyte basic protein (MOBP) significantly increase in their expression upon 9cRA treatment, suggesting that my initial hypothesis partly holds true. However, RXRγ mainly binds in proximity of genes involved in cell cycle regulation and control of proliferation. Interestingly, two of the genes that significantly change in the qPCR after only 24 hours of 9cRA exposure are relevant to both the above processes. *Cdkn1b* encodes for the cyclin dependent kinase inhibitor p27, a protein important in influencing cell cycle progression through the G1 phase. It is often used as a cell cycle exit marker as its main function is to slow down or stop cell division. Its regulation following RXRγ activation suggests that RXRγ affects the expression of salient players in cell cycle regulation and progression. FGF is a proliferative and migratory signal for OPCs, and this is mediated by FGFRs (McKinnon et al., 1990; Barres et al., 1994; Bansal, 2002; Lindner et al., 2015). The decrease of FGFR4 mRNA upon RXRγ activation suggests that RXRγ may regulate proliferation by interfering with this signalling pathway, in this case by limiting the amount of receptor present to detect FGF. This makes sense in an *in vivo* setting, where GFs such as FGF are continually being produced by astrocytes and microglia, and one of the potential ways to induce differentiation in the presence of proliferative signals is by making the cell oblivious to such signals by controlling the expression of the receptors.

These observations, alongside considerations from the literature, have aided me in the development of a new hypothesis with regards to RXRγ and its influence on OPC differentiation, which will be assessed and discussed in the following section.

3.4 RXR activation regulates OPC proliferation

In Section 3.3 I have presented data showing that RXRγ HREs are proximal to genes involved in proliferation and cell cycle control. The potent effect NRs exert on the cell cycle has been of particular interest in the context of cancer therapy and prevention, where the use of retinoids and rexinoids has been studied for the development of cancer differentiation therapies (Altucci et al., 2007). Unlike chemotherapy, where the aim is to destroy fast-dividing cells, the goal of cancer differentiation therapy is to drive malignant cells into terminal differentiation. The challenge in developing these therapies is therefore to reactivate pathways that are usually bypassed or suppressed in tumorogenesis, restoring a normal cycle and guiding the cancer cell into either differentiation or apoptosis (Altucci et al., 2007). RAR and RXR have been the candidates of choice for such therapies, as ATRA has proven successful as a differentiation therapy for the treatment of acute promyelocytic leukaemia (APL). Using a combination of ATRA and chemotherapy, 70%-80% of patients with APL present long-term remission, compared to only 25%-30% of patients achieving the same result prior to the introduction of ATRA (Lengfelder et al., 2005). However, the potent side and teratogenic effects of retinoids and pan-retinoids has pushed the field to search for less injurious alternatives.

Rexinoids are less toxic than retinoids (Miller et al., 1997) and have been just as successful in the treatment of diverse cancers, presenting an attractive alternative to retinoids. The efficiency of 9cRA in decreasing the tumorogenic phenotype of cells has been demonstrated in a variety of *in vitro* and *in vivo* cancer models. For example, Gottardis et al. (1996) have shown that 9cRA decreases proliferation and increases differentiation of the human promyelocytic leukaemia cell line HL-60 *in vitro*. Instead, *in vivo* 9cRA treatment of mice transplanted with primary human squamous cell carcinomas resulted in complete tumour regression (Gottardis et al., 1996). Furthermore, the RXR ligand Bexarotene has been the first FDA approved synthetic RXR agonist to be used for the treatment of cutaneous T cell lymphomas (Kempf et al., 2003). As a consequence, in the past years RXR agonists have been at the centre of cancer research, in order to develop treatments for an array of diverse tumours and different aspects of tumour biology (Altucci et al., 2007; Sabnis et al., 2013; Huang et al., 2016; Kiss et al., 2017). However, the mechanism by which rexinoids induce a differentiated phenotype in primary cancer cells and cell lines is still unknown.

RXR has been reported to enhance the differentiation effects of other NRs such as PPAR (Tontonoz et al., 1997; Crowe and Chandraratna, 2004). This suggests synergism between the activation of different NRs, which could arise from activation of the individual RXR-PPAR heterodimer components, or from sequential activation of RXR and PPAR, with each NR involved in different aspects of the cell cycle and progenitor differentiation. Indeed, in

the obesity field it has been reported that RXR manipulation determines cell cycle arrest in the G0/G1 phase in adipocytes, further suggesting its role in cell cycle regulation (Nakatsuka et al., 2012). Taking into account the literature in both the cancer and metabolic fields, as well as the data obtained from the ChIP-seq experiment reported in the previous section, it is plausible to suggest that RXR may affect cell cycle regulation.

3.4.1 9cRA treatment decreases OPC proliferation in serum-free conditions

To determine whether RXR activation alone affects OPC proliferation I resorted to OPC isolation using MACS and serum-free cultures, as for the differentiation experiments in Section 3.2.3. I assessed the percentage of proliferating progenitors using EdU, a nucleoside analog of thymidine, which is incorporated by the dividing cell into the DNA during DNA replication. Thus, EdU labels all cells that are in S phase of the cell cycle, as this is when active DNA synthesis occurs. This experiment was carried out in both presence and absence of GFs, these being strong drivers of OPC proliferation whose absence could mask the potential effect elicited by the treatments being assessed (Woodruff et al., 2004; Murtie et al., 2005). Following the same rationale of the experiments in Section 3.2.3, T3 was used as the positive control as it has been shown to induce OPC differentiation at the expense of proliferation in both GF presence and absence (Barres et al., 1994).

Figure 3.17A shows the effect of GF presence and absence on OPC proliferation in control conditions. The percentage of proliferating OPCs in GF presence was on average 62.9% $\pm$ 5.7% 24 hours after isolation and remains constant for the 96 hours following OPC isolation (ordinary one-way ANOVA, n=3). Instead, when GFs are removed there was a constant and statistically significant decrease in the number of proliferating OPCs (ordinary one-way ANOVA followed by Tukey's multiple comparisons test where there was significance, $p<0.0001$, n=3). The percent of proliferating OPCs almost halves in the first 96 hours, where the percentage of EdU^+ OPCs decreased from an average of 51.56% $\pm$ 2.07% at 24 hours, to an average of 27.07% $\pm$ 3.05% 96 hours after GF removal. This indicates that the OPCs in culture respond as expected to GF presence and removal. The effect observed in control conditions following GF removal was replicated in the presence of both 9cRA and T3 treatments as illustrated in Figure 3.17B, where each treatment shows a decrease in OPC proliferation over time (ordinary one-way ANOVA followed by Tukey's multiple comparisons test where there was significance, $p<0.0001$ for vehicle control and 9cRA, $p<0.001$ for T3, n=3). However, when the treatments are compared at the same time point, no difference in the percent of proliferating OPCs was observed. This suggests that the decrease

in proliferation in GF absence is due to the effect induced by GF removal, rather than the treatments *per se*. Instead, the effect of RXR activation on OPC proliferation became immediately apparent in the presence of GFs. After only 24 hours of 9cRA exposure, there was a statistically significant decrease in the percentage of EdU^+ OPCs compared to the vehicle control, which decreased by approximately 20%, is observed (Figure 3.17B). The drop in proliferation of 9cRA treated progenitors exceeds the one observed in T3 treated cells after 48 hours, suggesting a stronger effect of 9cRA on proliferation compared to T3 at these early time points. However, over time both vehicle control and T3 treated cells caught up, with the latter exceeding the decrease in OPC proliferation presented by the 9cRA treatment (ordinary one-way ANOVA followed by Tukey's multiple comparisons test where there was significance, n=3).

In Section 3.2.3 the use of CNPase as a marker for differentiation did not show any significant difference between 9cRA exposure and control conditions. ChIP-seq clearly showed that this differentiation marker, alongside other markers such as MBP, PLP, MAG and MOG, are not controlled by RXRγ, therefore this time I used cell morphology as an indicator of whether RXR activation affected early OPC differentiation. This method had been previously adopted by Huang et al. (2010a) to quantify OPC differentiation. After 6 days of *in vitro* treatment, progenitors were classified as either bipolar or multipolar based on the morphology obtained via O4 staining as shown in Figure 1.3. In both absence and presence of GFs, the percentage of bipolar progenitors remains constant across treatments, whilst T3 results in a significant increase in the percent of multipolar cells, with p=0.0118 in GF presence and p=0.0052 in GF absence (Figure 3.17C). Instead, there is no significant difference in the percentage of multipolar cells in 9cRA and control conditions (ordinary one-way ANOVA followed by Tukey's multiple comparisons test where there was significance, n=3). The above demonstrates that RXR activation alone affects OPC proliferation but not differentiation, as shown in both Figures 3.17 and 3.6, where no changes in percentage of $CNPase^+$ cells or multipolar morphology are observed. This reinforces the concept that proliferation and differentiation are highly coordinated, yet independent processes.

The effect of the RXR inhibitor HX531 (HX) was also tested in these conditions. However, these experiments were carried out at a different time, and due to technical issues with the A2B5 antibody following changes to its manufacturing process by the provider, a lack of specificity and suboptimal purity of the MACS isolations affected the downstream results. Consequently, OPCs *in vitro* were not responding as expected in control conditions. Due to the above, this data is not presented in this thesis.

OPCs have the ability of producing endogenous 9cRA. This is carried out by the enzyme aldehyde dehydrogenase 1A1 (ALDH1A1) whose function is the conversion of lipid alde-

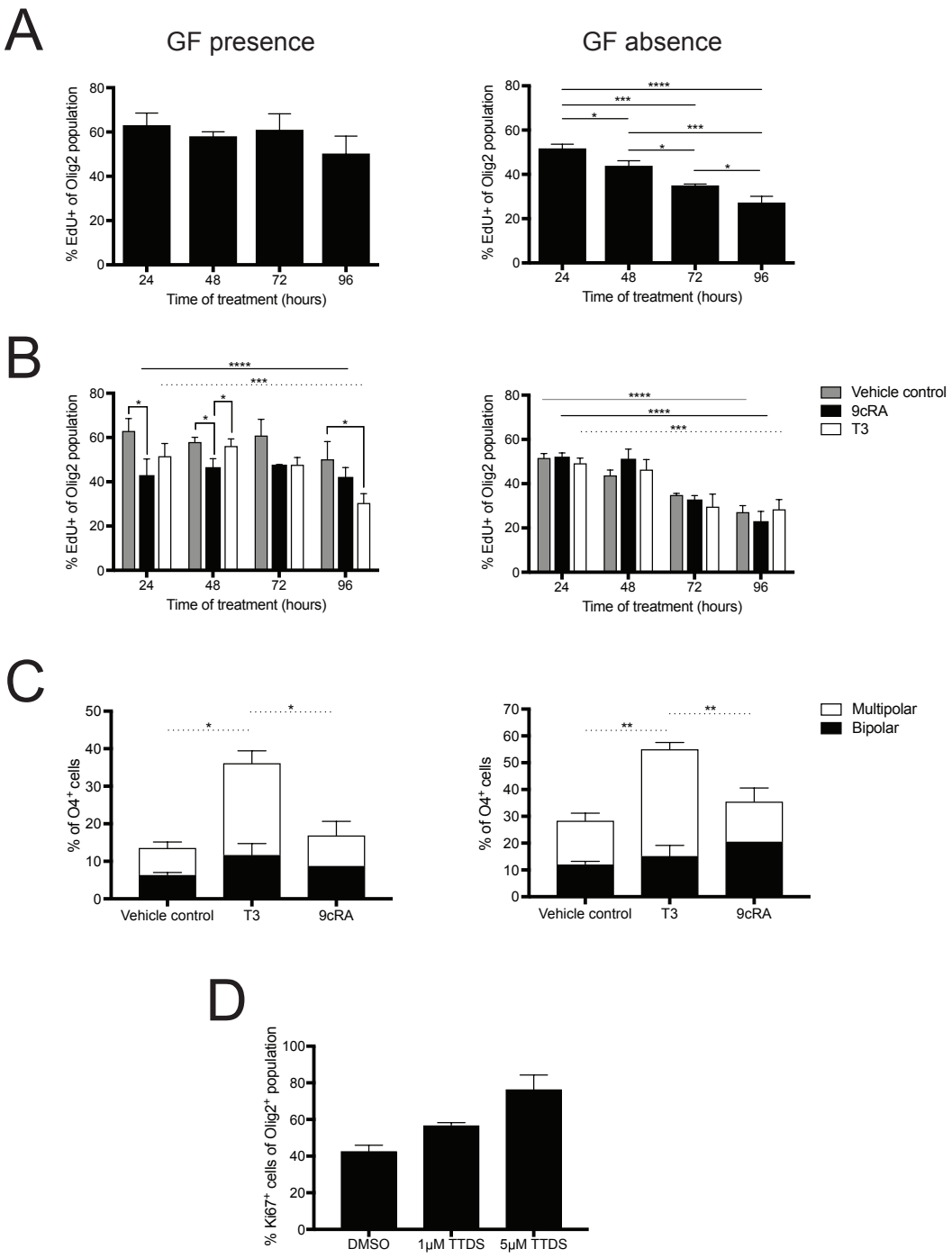


Fig. 3.17 Effect of 9cRA on the proliferation of MACS sorted rat primary OPCs cultured in serum-free conditions. (A) In control conditions GF presence maintains OPCs in constant proliferation, whilst their removal decreases their proliferation. (B) Effect of 9cRA and T3 on OPC proliferation in GF presence and absence. (C) OPC differentiation assessed via morphology of O4 stain. Only T3 shows increased differentiation. (D) Inhibition of ALDH1A1 via TTDS shows increased OPC proliferation (biological n=2). All data was analysed using ordinary one-way ANOVA followed by Tukey's multiple comparison test where significance was reached. All graphs are presented as mean ± SEM, biological n=3 unless specified (*p≤0.05, **p≤0.01, ***p≤0.001, ****p<0.0001).

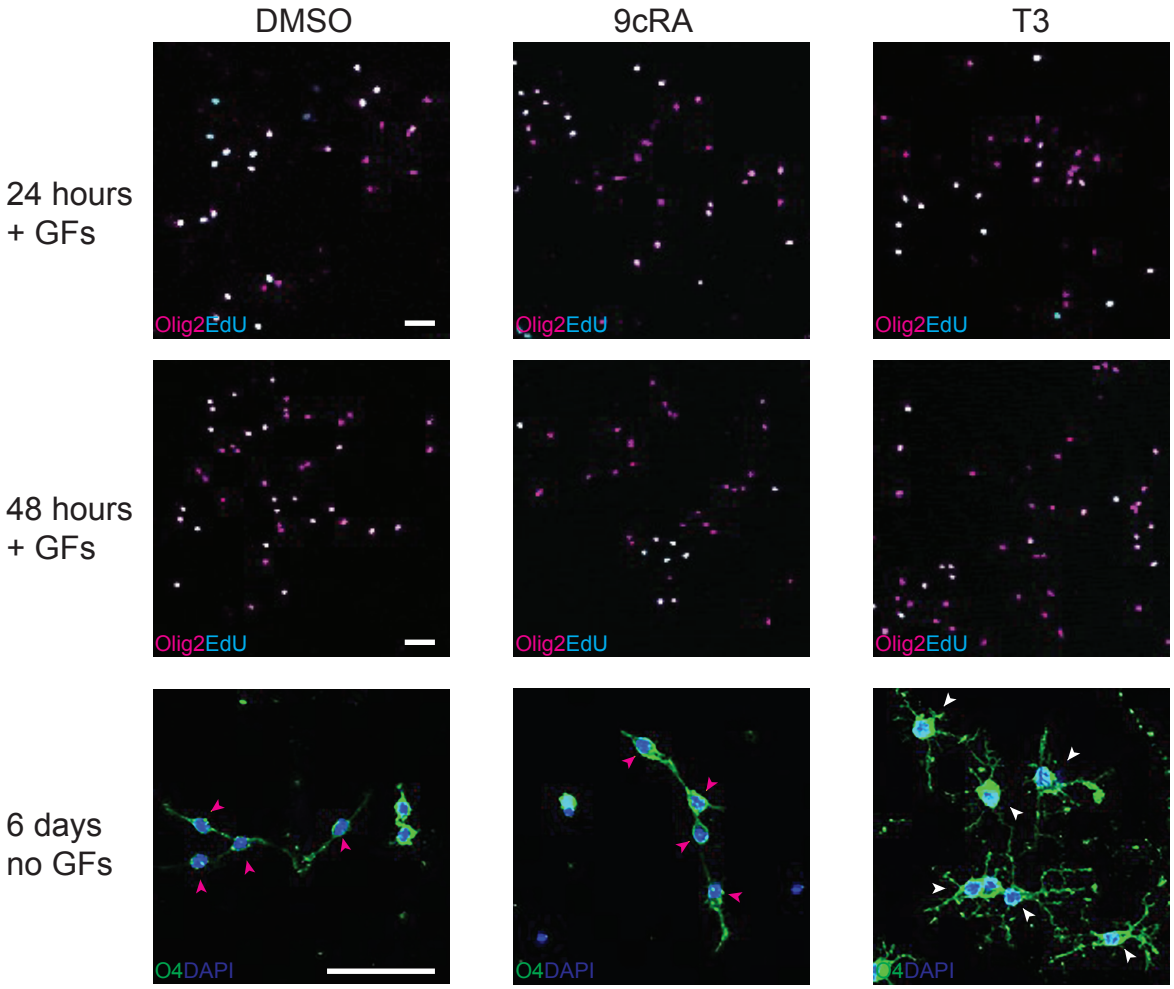


Fig. 3.18 Immunocytochemistry of 9cRA effect on OPC proliferation and morphology. Representative pictures for the results in Figure 3.17A, B and C.The top two rows show EdU labelled OPCs in the three conditions after 24 and 48 hours of exposure in GF presence. The bottom row shows the morphology after six days of treatment exposure. Pink arrows show progenitors with a bipolar morphology and white arrows show progenitors with a multipolar morphology. Bipolar progenitors are in the early stages of the lineage, whilst multipolar morphology suggests that the cells are beginning to differentiate. Scale bar = 50μm.

hydes to lipid carboxylic acids. It is therefore possible to use the substrate 9-cis retinal and endogenously convert it to 9cRA (Paterson et al., 2013). ALDH1A1 appears in the top 500 unique gene signature of OPCs compared to newly formed and myelinating oligodendrocytes shown in Figure 3.16A, suggesting an important role for 9cRA in OPC biology. To determine whether complete absence of 9cRA affected OPC proliferation, I treated MACS sorted OPCs with the ALDH1A1 inhibitor tetraethylthiuram disulfide (TTDS) for 6 days *in vitro*. The OPCs were then fixed and stained for the proliferation marker Ki67. It is possible to observe in Figure 3.17D that TTDS treatment increases OPC proliferation in a dose dependent manner. In control conditions an average of 42.66% (SD=3.271) of OPCs were Ki67$^+$, nearly doubling upon 5μM TTDS treatment to an average of 76.36% (SD=7.903). The number of CNPase$^+$ cells did not change significantly (DMSO: mean=12.79% $\pm$ 1.356%, 1μM TTDS: mean=19.38% $\pm$ 15.64%, 5μM TTDS: mean=6.414% $\pm$ 7.592%). Statistical analysis could not be carried out on these set of experiments due to the low number of biological replicates (n=2). However, these initial observations suggest that in the absence of the RXR agonist 9cRA, the number of proliferating OPCs increases, further supporting the hypothesis that RXR modulation regulates OPC proliferation without affecting differentiation. The ALDH1A1 experiments were kindly carried out by Alerie Guzmán de la Fuente.

3.4.2 Testing the effect of RXR on the cell cycle and the exit marker p27

Following the observation that the level of p27 mRNA increases and that OPC proliferation *in vitro* decreases after 24 hours of 9cRA exposure (*Cdkn1b* in Figure 3.16E), I sought to understand how RXR activation governs cell cycle dynamics. Based on the role of p27 in the literature (Fero et al., 1996, 1998; Goukassian et al., 2001; Sharma et al., 2011), I hypothesised that there are two possibilities as to why I observe a decrease in proliferation:

1. RXR activation promotes cell cycle exit;
2. RXR activation lengthens the G1 phase of the cell cycle, thereby delaying entry into S phase.

Either way, the ultimate effect is the decrease or the slowing down of OPC proliferation. This would explain why less progenitors are found in S phase upon 9cRA treatment compared to control (Figure 3.17B). Once again, the following results were carried out during the previously mentioned technical issues with the A2B5 antibody used to isolate OPCs and so will need to be repeated in order to properly assess the ultimate outcome. However, they are presented in this thesis as an example of the approach that I would have used to determine how RXR manipulation influences the cell cycle.

In Figure 3.16E it is clear that the cell cycle exit marker p27 is significantly higher in 9cRA treated OPCs compared to the vehicle control. However, so far this has only been observed at the mRNA level and in order for p27 to exert its function it needs to be translated into protein. I therefore decided to assess the protein level of p27 in 9cRA and HX treated OPCs using western blot. OPCs were MACS sorted and treated *in vitro* for 24 or 48 hours in both presence and absence of GFs. Only the results for 24 hours are shown in Figure 3.19A due to the previously discussed compromised purity of the isolation. From the results, it appears that in GF presence there is no difference in the protein level of p27 between the different treatments, whilst in the absence of GFs it seems to decrease for 9cRA compared to the vehicle control, and decrease even more upon RXR inhibition. Despite the unexpected result, more technical replicates are required in order to carry out proper statistical analysis and draw appropriate conclusions. Another, yet less reliable method for determining changes in p27 levels is by determining cells with either low or high p27 fluorescence upon immunocytochemistry. This has been adopted and reported by groups such as the Casaccia laboratory as a method to estimate changes in protein expression (Scaglione et al., 2018). I therefore carried out immunocytochemistry for p27 on OPCs treated for 24 or 48 hours with either 9cRA or HX. Figure 3.19B shows that at both time points there is no difference in the percentage of high or low p27 expressing OPCs across treatments, suggesting no changes in the expression level of p27 upon RXR activation or inhibition compared to control conditions (ordinary two-way ANOVA, n=3). However, immunofluorescence level remains a tenuous method for determining overall protein amount as it is not a precise quantitative measurement. Additionally, I sought out to assess the protein levels of cyclins important to the G1-S phase transition, such as cyclin D1 and cyclin E1. I carried out western blot for the two cyclins on OPCs exposed for 6, 12 and 24 hours to 9cRA, for which the results are shown in Figure 3.19C. No statistically significant differences were reported in the protein level of either cyclins in 9cRA treated OPCs compared to vehicle control. The time of exposure to 9cRA also shows no changes in the level of these two cyclins. One of the reasons for this result could be that the reported time points are too early in order to observe an effect at the protein level. Furthermore, these OPCs were treated in the absence of GFs, and it may well be that this masks any 9cRA effect as for the experiments reported in Figure 3.17. These experiments should therefore be repeated in the appropriate conditions in order for the results to be adequately interpreted.

To assess the second possibility of whether RXR activation induces G1 lengthening, I adopted a flow cytometry approach to determine whether there is a change in the OPC distribution between the different cell cycle stages upon 9cRA treatment. This method is based on the use of a nuclear stain such as PI, which intercalates in the DNA bases of cells.

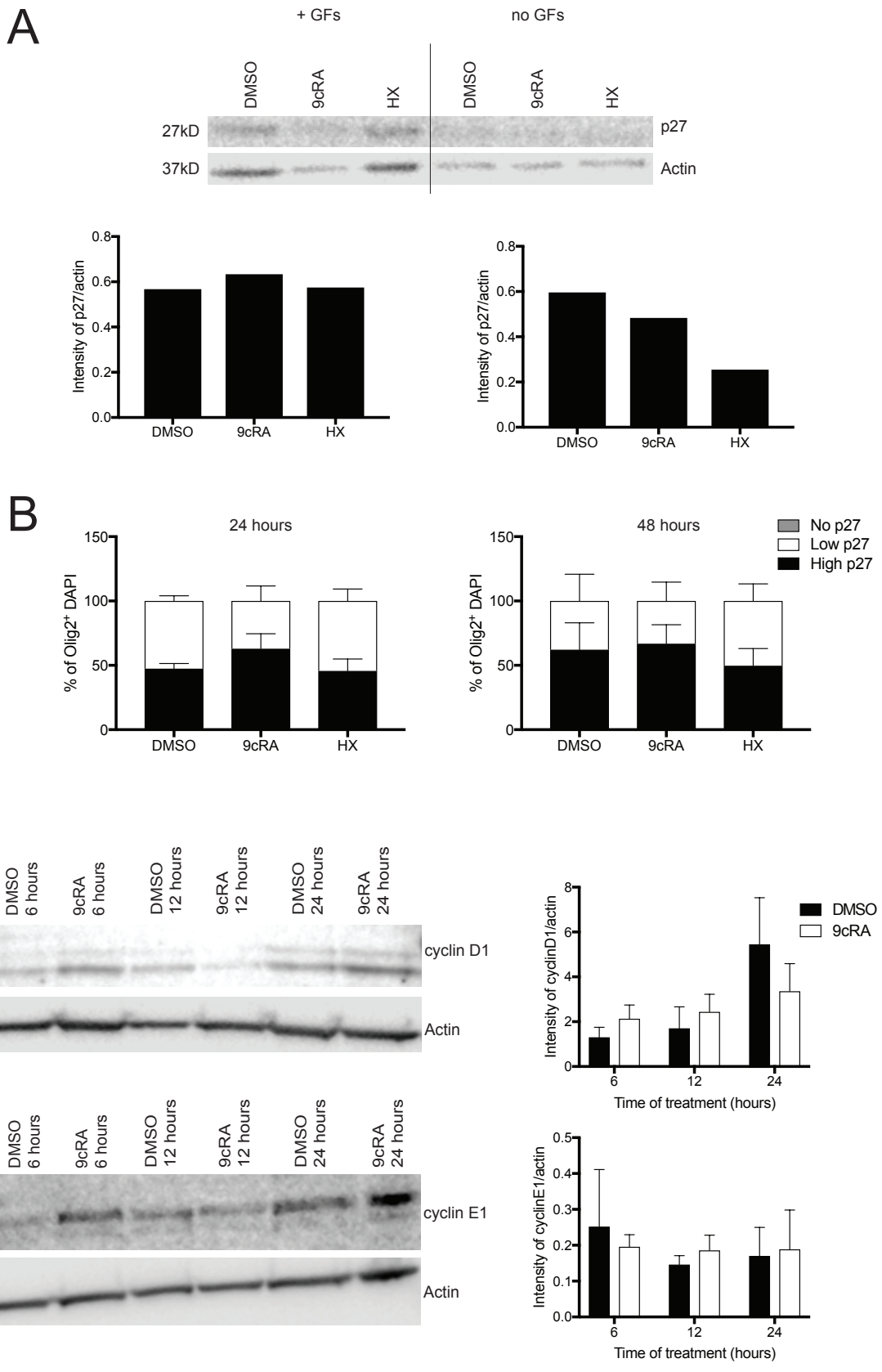

A
+ GFs
no GFs
DMSO
9cRA
HX
DMSO
9cRA
HX
27kD
p27
37kD
Actin
Intensity of p27/actin
0.8
0.6
0.4
0.2
0.0
DMSO
9cRA
HX
Intensity of p27/actin
0.8
0.6
0.4
0.2
0.0
DMSO
9cRA
HX
B
24 hours
48 hours
No p27
Low p27
High p27
% of Olig2+ DAPI
150
100
50
0
DMSO
9cRA
HX
% of Olig2+ DAPI
150
100
50
0
DMSO
9cRA
HX
C
DMSO 6 hours
9cRA 6 hours
DMSO 12 hours
9cRA 12 hours
DMSO 24 hours
9cRA 24 hours
35kD
cyclin D1
37kD
Actin
DMSO 6 hours
9cRA 6 hours
DMSO 12 hours
9cRA 12 hours
DMSO 24 hours
9cRA 24 hours
50kD
cyclin E1
37kD
Actin
Intensity of cyclinD1/actin
8
6
4
2
0
6
12
24
Time of treatment (hours)
DMSO
9cRA
Intensity of cyclinE1/actin
0.5
0.4
0.3
0.2
0.1
0.0
6
12
24
Time of treatment (hours)

Fig. 3.19 Changes in cell cycle markers upon RXR activation. These experiments were unfortunately carried out following a change to the manufacturing process of the A2B5 antibody used for the MACS isolation of primary OPCs. Due to this, a lack of specificity and suboptimal purity of the isolation compromised the following results. They should therefore not be taken as absolute and the experiments will need to be repeated once the issue is resolved. (A) Western blot gel and quantification of p27 expression in OPCs after 24 hours of treatment exposure (biological n=1). (B) p27 quantification via immunocytochemistry resulted in no significant difference in the percentage of $Olig2^+$ cells expressing p27. Data analysed using ordinary two-way ANOVA. All graphs presented as mean ± SEM, biological n=3. (C) Western blot gels and quantification of cyclins D1 and E1 in OPCs. Results show no significant difference in cyclin D1 expression between DMSO and 9cRA after 6, 12 and 24 hours of treatment exposure, nor between each time point for the same treatment. There seems to be no difference in the level of cyclin E1 expression either. Data analysed using unpaired student t test and ordinary one-way ANOVA. All graphs presented as mean ± SEM, biological n=3 for cyclin D1, biological n=2 for cyclin E1. However, the purity of the OPCs isolated for these experiments was not optimal, therefore they will need to be repeated with the appropriate OPC purity.

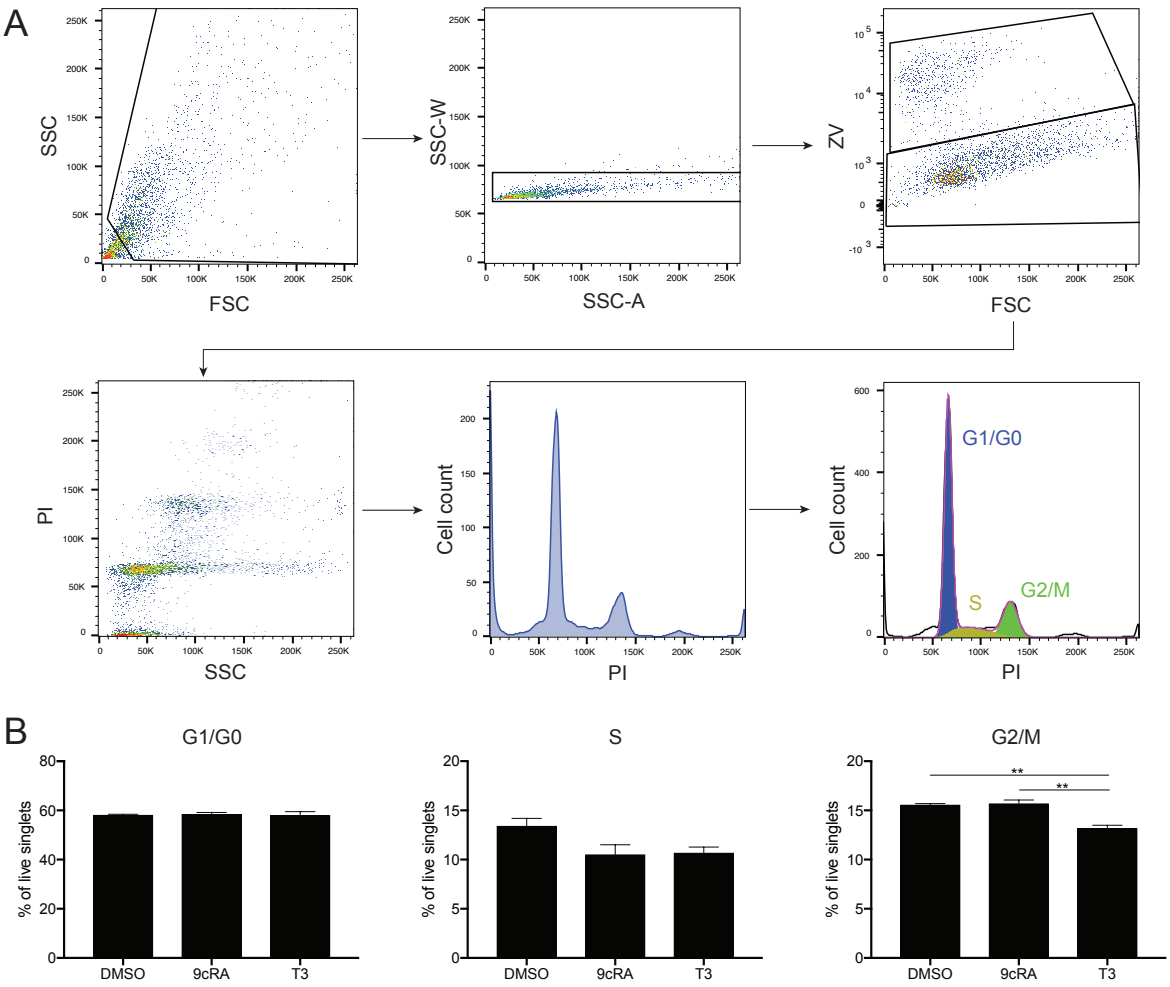


Fig. 3.20 OPC distribution across cell cycle stages upon RXR activation. These experiments were unfortunately carried out following a change to the manufacturing process of the A2B5 antibody used for the MACS isolation of primary OPCs. Due to this, a lack of specificity and suboptimal purity of the isolation compromised the following results. They should therefore not be taken as absolute and the experiments will need to be repeated once the issue is resolved. Panel (A) illustrates the gating strategy adopted in order to determine the progenitor distribution. The forward and side scatter are initially used to determine cells. Of all the singlets only the live cells will be assessed, therefore the forward scatter and ZV stain aid in the exclusion of the dead cell population which is high in ZV. The live singlets are then plotted using the side scatter and PI intensity. The three populations observed are the progenitors distributed between the G1/G0, S and G2/M. The PI intensity is plotted against the cell count as a histogram to which the Dean-Jett-Fox model is applied, an algorithm which allows for the correct division of the progenitors across the three stages illustrated in three different colours (Fox, 1980). (B) The results obtained from the application of the Dean-Jett-Fox model are used for statistical analysis. No differences are observed in the distribution of progenitors across G1/G0 or S phases for the three treatments. However, T3 treated OPCs present a significant decrease in the numbers found in G2/M compared to the other two treatments ($p=0.0013$). Therefore, T3 could block or slow down progression through G1/G0 and/or S, or accelerate G2/M progression. All data was analysed using ordinary one-way ANOVA followed by Tukey's multiple comparisons test where there was significance. All graphs are presented as mean ± SEM, biological n=3.

As DNA amount and conformation are different between the G1/G0, S and G2/M phases, PI staining allows to grossly group the cells into the above three stages, presenting a final distribution of the overall sample tested. Based on the observation in Section 3.17, I isolated OPCs via MACS and treated them *in vitro* with DMSO, 9cRA or T3 for 48 hours in GF presence. After 48 hours the progenitors were detached, stained for cell death and PI as explained in Section 2.2.12 and taken to the flow cytometer.

The gating strategy is shown in Figure 3.20A. To obtain cells, the progenitors are initially plotted using the forward and side scatter. Once the singlets are obtained, ZV is used to determine which cells are dead, allowing for the exclusion of these cells from the analysis. Once the live singlets are obtained, PI is plotted against the side scatter, and based on the complexity and PI intensity the cells are grouped into the three broad stages of the cell cycle mentioned above. To determine the percentage of cells in each phase the Dean-Jett-Fox model found in FlowJo is used (Fox, 1980). This is an algorithm that allows for the precise classification of the cells into each phase. Analysis of the first set of results revealed no statistically significant difference in the number of cells in each stage upon 9cRA treatment compared to the control (3.20B). On the other hand, T3 resulted in a significant decrease in the number of cells in the G2/M phase compared to control and 9cRA (ordinary one-way ANOVA followed by Tukey's multiple comparisons test where there was significance, p=0.0013, n=3). The decrease in number of cells in G2/M phase could mean that T3 treatment slows down the progression through one or both of the other stages, or that it accelerates progression through G2/M. Either way a shift in the distribution of cell numbers in the other stages may also be expected as a consequence. However, due to the compromised purity of the isolation, these experiments need to be repeated in order to determine the true effect of 9cRA and T3 on the OPC cell cycle. Furthermore, the number of cells obtained from each treatment varies substantially, meaning that there were great discrepancies in the size of the samples analysed. In this set of results the 9cRA samples were the ones with the least progenitors analysed, with half as many cells as the vehicle control (results not shown). This could lead to an underrepresentation of the sample and the results could be compromised. It is therefore necessary to improve the collection of the progenitors from *in vitro* conditions in order to obtain samples with a high and equal number of cells to analyse.

3.4.3 Discussion

The data presented in this section shows that RXR activation *in vitro* results in a decrease in proliferation. Interestingly, this occurs in serum-free conditions and GF presence, suggesting that:

1. the true effect of RXR activation affects OPC proliferation, which could consequently impact on OPC differentiation in the presence of other NR ligands as observed in Figure 3.7C;

2. RXR activation has a similar outcome to GF removal as the effect of 9cRA on proliferation is masked in the absence of GFs, while it is obvious in their presence.

From the ChIP-seq results and the functional data presented above, it is clear that RXR activation affects OPC proliferation *in vitro*. Further confirmation comes from the cancer biology field where RAR and RXR have been used to develop cancer differentiation therapies as previously discussed. As the main aim of the therapy is to make malignant cells exit the cell cycle in order for differentiation to occur, it is plausible to assume that RXR activation influences cell cycle exit. Despite the cell-specific effects of NRs, their ubiquitous expression and conservation across cell-types makes it tempting to propose that they probably control similar cellular programs in different cell types. In this case, RXR may be involved in controlling proliferation and cell cycle exit. Furthermore, Huang et al. (2010a) showed that 9cRA treatment *in vivo* accelerates remyelination in aged rats, which was initially interpreted as an induction of OPC differentiation based on *in vitro* experiments carried out on mixed glia isolated OPCs. However, remyelination was also assessed in RXRγ knockout mice, where remyelination was delayed but not completely inhibited (Huang et al., 2010a). Moreover, the data that I have presented in Section 3.2.3 and Figure 3.17C clearly show that RXR activation does not lead to an increase in the differentiation of MACS isolated OPCs, unless other NR ligands are also present. Instead, RXR activation does lead to a decrease in the proliferation of MACS isolated OPCs, without affecting their differentiation (Figure 3.17B and C). Taken together, these results suggest that OPC differentiation and remyelination are not dependent on RXRγ *per se*, but are still influenced by its activation and absence. Given that RXRγ associates to genes involved in OPC proliferation as reported in the ChIP-seq in Section 3.3, and that 9cRA treatment of MACS isolated OPCs leads to an increase in p27 mRNA as shown in the qPCR results in Figure 3.16E, the ability of RXRγ to influence the cell cycle could be a plausible explanation for this phenomenon, as cell cycle exit or G1 lengthening would facilitate OPC differentiation and consequently remyelination, but not necessarily depend on it.

Previous work in the laboratory did not observe this same effect on proliferation upon 9cRA treatment of mixed glia derived OPCs. This is probably because the presence of serum saturates the system with pro-differentiation factors as discussed in Section 3.2.3 and observed in Figure 3.7, resembling an environment lacking GFs or overriding the proliferative effects of said mitogens. As the consequence of RXR activation on OPC proliferation is ob-

vious only in the presence of GFs (Figure 3.17B), it does not come as a surprise that previous studies involving mixed glia isolation did not discern the same observations.

In this Section, I have described the ability of RXR activation to decrease *in vitro* OPC proliferation without affecting differentiation. The ChIP-seq results reported RXRγ to bind in close proximity of the cell-cycle exit marker p27. Subsequent functional validations showed an increase in p27 mRNA after 24 hours of 9cRA exposure compared to the control. Therefore, I initially assessed whether the decrease in proliferation is dependent on cell cycle exit. I tested whether the changes in p27 expression observed via qPCR translated at the protein level, however due to technical issues these experiments need to be repeated. Consequently, it would be interesting to determine whether p27 acts on OPCs by promoting cell cycle exit, or whether it lengthens their G1 phase.

During the cell cycle, the length of the G1 phase has been shown to be important in fate determination of pluripotent stem cells (PSC) (Sela et al., 2012; Coronado et al., 2013; Calder et al., 2013; Pauklin and Vallier, 2014; Boward et al., 2016), the implications of which are further discussed in the final discussion (Chapter 4). G1 lengthening, as opposed to cell cycle exit, would result in a more subtle effect rather than a complete impairment, which could describe the delay in remyelination observed by Huang et al. (2010a) in *RXRγ null* mice. To determine whether cell cycle stages were affected upon RXR activation I initially carried out flow cytometry using PI, which allows to determine whether there are changes in the distribution of the cell cycle stages. This initial analysis did not yield reliable results due to the technical issues imposed by the new anti-A2B5 antibody batches used for MACS sorted OPCs. However, this flow cytometry experiment can be improved by incorporating the use of antibodies against cyclins specific to each stage, as this would allow for a more precise distinction of which phase each OPC is in. Furthermore, the use of the fluorescence ubiquitination-based cell cycle indicator (FUCCI) is an interesting avenue to study the transition from G1 to S phase both *in vitro* and *in vivo* (Sakaue-Sawano and Miyawaki, 2014). FUCCI uses ubiquitination oscillators involved in the control of cell-cycle transition which genetically express fluorescent probes (Sakaue-Sawano and Miyawaki, 2014). This allows for a dynamic colour change, from red to yellow to green, displaying the progression through the cell cycle, resulting in a more efficient technique to determine cell cycle progression.

The above would only be a starting point for the study of cell cycle dynamics, since it is not possible to determine the length and kinetics of the cell cycle phases from flow cytometry alone, which is the ultimate goal of this study. Instead, this requires prior knowledge of the overall length of the cell cycle via pulse labelling experiments which, in combination with PI in flow cytometry, can be used to determine the approximate length of each stage. Ultimately, whether RXR manipulation can influence cell cycle dynamics as opposed to dif-

ferentiation would be an interesting and relevant question with regards to stem cell ageing. This is because young and aged progenitors have different cell cycle dynamics despite being the same cell-type. Indeed, the OPC cell cycle has been reported to lengthen with increasing age and this correlates with a decline in oligodendrocyte generation in homeostasis (Young et al., 2013), as well as with an impairment in remyelination (Sim et al., 2002). Therefore, altered cell cycle dynamics with ageing may be part of the reason as to why OPCs differentiate less efficiently. Considering the observations reported above and that RXR activation accelerates remyelination in aged animals, it is plausible to think that it may do so by affecting the cell cycle dynamics of the aged OPC. However, whether NR expression and their ability to respond to ligands changes in aged OPCs, and whether these changes are detrimental to remyelination, still needs to be understood.

3.5 Nuclear receptors in ageing OPCs

One of the major hallmarks of chronological cellular ageing is the accumulation of epigenetic changes. These include alterations in DNA methylation patterns, histone modifications and chromatin remodelling. The latter is particularly interesting to NR biology as their main role is to orchestrate chromatin remodelling in order to control gene transcription (Egea et al., 2000; Aranda and Pascual, 2001; Evans and Mangelsdorf, 2014). It has in fact been observed that the expression level of enzymes and proteins involved in chromatin remodelling, such as heterochromatin protein 1α (HP1α), polycomb complexes and the NuRD complex, decrease with ageing (Pegoraro et al., 2009; Pollina and Brunet, 2011).

The loss of such proteins inevitably induces alterations in the chromatin architecture of the cell characterised by a loss of heterochromatin. In humans it has been observed that mutations in lamins cause the development of the premature ageing diseases Hutchinson-Gilford Progeria Syndrome and Werner Syndrome (Shumaker et al., 2006; Zhang et al., 2015b). Lamins are the major components of the nuclear lamina which not only structurally supports the shape of the nucleus, but also acts as a docking site for heterochromatin. Indeed, the cells of these patients also present altered nuclear morphology such as changes in chromosome centromeres, a loss in the heterochromatin mark H3K9me3, as well as delocalisation or lack of proteins related to heterochromatin, such as HP1α, pericentric satellite III repeat and the EZH2 methyltransferase, consequently leading to altered methylation marks (Shumaker et al., 2006). These findings point towards a disruption of the chromatin structure in cells displaying an aged phenotype, a conclusion which has been reinforced by the observation of spontaneous chronological ageing in various animal models and humans (Haithcock et al., 2005; Scaffidi and Misteli, 2006; Brandt et al., 2008). For example, flies with impaired HP1α present a reduced lifespan, whilst its overexpression extends longevity and delays age-related muscular deterioration (Larson et al., 2012). Changes in chromatin structure inevitably lead to alterations in transcription. Indeed, quantification of mRNA levels of cardiomyocytes from young and old mice revealed an increase in the transcriptional noise in aged cells compared to young (Bahar et al., 2006). More recently, single cell RNA-seq analysis of $CD4^+$ T cells revealed an increase in transcriptional heterogeneity in the cells of older mice compared to young (Martinez-Jimenez et al., 2017). Additionally, the production and processing of mRNAs, both protein coding and non-coding, are impaired in aged cells compared to young (Harries et al., 2011; Boulias and Horvitz, 2012; Lee et al., 2016; Rodríguez et al., 2016).

Transcriptional regulation is at the basis of a cell's ability to respond adequately to environmental signals in order to carry out its function and survive. Therefore, its impairment in ageing can have detrimental consequences. This is particularly true for stem cells which

need to be able to respond correctly to their environment in order to self-renew or differentiate into the required cell type, making their alterations in chromatin structure arguably more damaging than those of post mitotic cell types. NRs are key players in chromatin remodelling and transcriptional regulation. However, their involvement or impairment in the context of stem cell ageing has not been researched extensively. Indeed, unpublished work from the Franklin laboratory carried out by Björn Neumann has shown that aged OPCs *in vitro* do not respond to conventional signals such as T3, ATRA and 9cRA as efficiently as young OPCs. This suggests that ageing decreases the inherent differentiation potential of adult OPCs. The inability of aged OPCs to respond to external signals can ultimately lead to the impairment in remyelination that is observed in ageing scenarios (Shields et al., 1999). A particularly important hallmark of stem cell ageing is increased cellular senescence, characterised by cell cycle arrest compromising stem cell function. This has been observed in numerous stem cell niches (Janzen et al., 2006; Nishino et al., 2008; Signer et al., 2008; Sousa-Victor et al., 2014). As with differentiation, this could occur as a consequence of the progenitor's inability to respond to signals that promote cell cycle progression. Furthermore, part of the ageing phenotype appears to be dependent on the circulatory system where NR ligands are transported. Heterochronic parabiosis experiments have revealed that factors in the circulation can modulate ageing organs and rejuvenate them, including the brain (Ruckh et al., 2012). Due to the above, whether NR pathways and networks are altered with ageing is an interesting question, as these transcription factors bridge extracellular signals with transcriptional regulation.

3.5.1 NR expression in ageing OPCs

In order to assess changes at the protein level, I carried western blot on MACS sorted OPCs lysed immediately after the isolation. I isolated OPCs from 3, 10 and over 18 months old rats, which are the equivalent of young, middle-aged and aged humans, whilst neonatal OPCs were used as controls. The results are shown in Figure 3.21A. Statistical analysis was carried out only for VDR and LXRα, as only one or two biological replicates were collected for the other NRs assessed. In both cases, the difference in expression between the various ages was not statistically significant, suggesting no changes in the level of expression of the receptors (ordinary one-way ANOVA, n=3). However, western blot is a semi-quantitative technique, whereby often the variability imposed by the technique is too big to yield significance. More precise techniques such as quantitative mass spectrometry are preferred. However, as reported in Section 3.1, NRs are expressed at levels below the limit of detection of the mass spectrometry technique used.

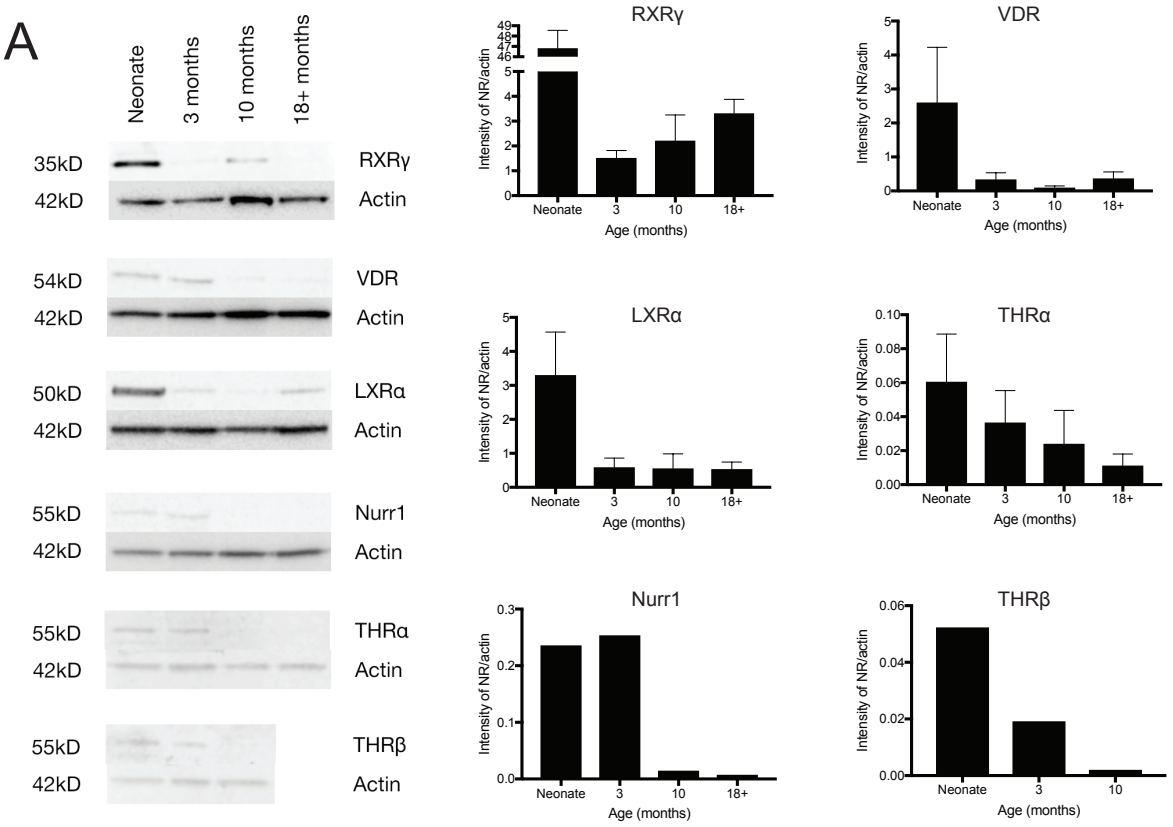
Despite the above, it is possible to observe that the NR expression decreases after development. Furthermore, their expression continues to be decrease in adulthood and ageing. The levels of THRα, THRβ and Nurr1 decrease between 3 and 18 months of age. It would be interesting to collect more replicates in order to determine whether this decrease is statistically significant. These observations are supported by unpublished results from the lab yielded from the RNA-seq comparison of young and aged rat OPCs carried out by Roey Baror. The volcano plot is shown in Figure 3.21B. Almost all the NRs detected by the sequencing are grouped in the right side of the plot, suggesting that their expression at the RNA level is lower in aged OPCs compared to young. THRα and β are present in this plot, giving further confidence that the patterns observed in the western blot analysis may indeed be true.

The volcano plot shows only one NR to increase at the RNA level in aged OPCs compared to young: RXRα. When looking at RXRγ expression at the protein level in Figure 3.21A, it appears that its expression at the protein level follows an increasing pattern between 3 and 18 months of age. Despite these observations require further validation, they perhaps suggest that RXRs are the only NRs among the ones assessed so far that tend to increase in their expression with increasing age, whilst the others decrease or remain unchanged.

3.5.2 Discussion

The preliminary data presented in this section suggests that there could be a change in the expression level of some NRs as OPCs age. Despite this being preliminary data that requires further validations and functional interpretation, it would be interesting to understand if these changes are reversible. For example, it is known that the expression of NRs such as VDR is often controlled via their ligands (Carrillo-López et al., 2008). Therefore the presence of the cognate ligand itself induces expression of the receptor. Consequently, it would be interesting to understand whether treating aged OPCs, where a decrease in receptor expression is observed, with the relevant ligand induces an increase in receptor expression. This would aid in understanding whether changes in NR expression with ageing are normal due to the necessities of the changing cell, or whether they have a detrimental nature and may be involved in the ageing phenotype. By treating OPCs of different ages *in vitro* it would be possible to correlate changes in NR expression observed via western blot with the cellular response to ligand presence.

Additionally, a decrease in NR ligands such as vitamin D and TH has been observed in ageing humans (Meehan and Penckofer, 2014; Hershman et al., 2015; Gesing, 2015). This is an interesting observation as such factors are blood-borne, and heterochronic parabiosis experiments have shown that aged animals remyelinate more efficiently when sharing the


A
Neonate
3 months
10 months
18+ months
35kD
42kD
RXRγ
Actin
54kD
42kD
VDR
Actin
50kD
42kD
LXRα
Actin
55kD
42kD
Nurr1
Actin
55kD
42kD
THRα
Actin
55kD
42kD
THRβ
Actin
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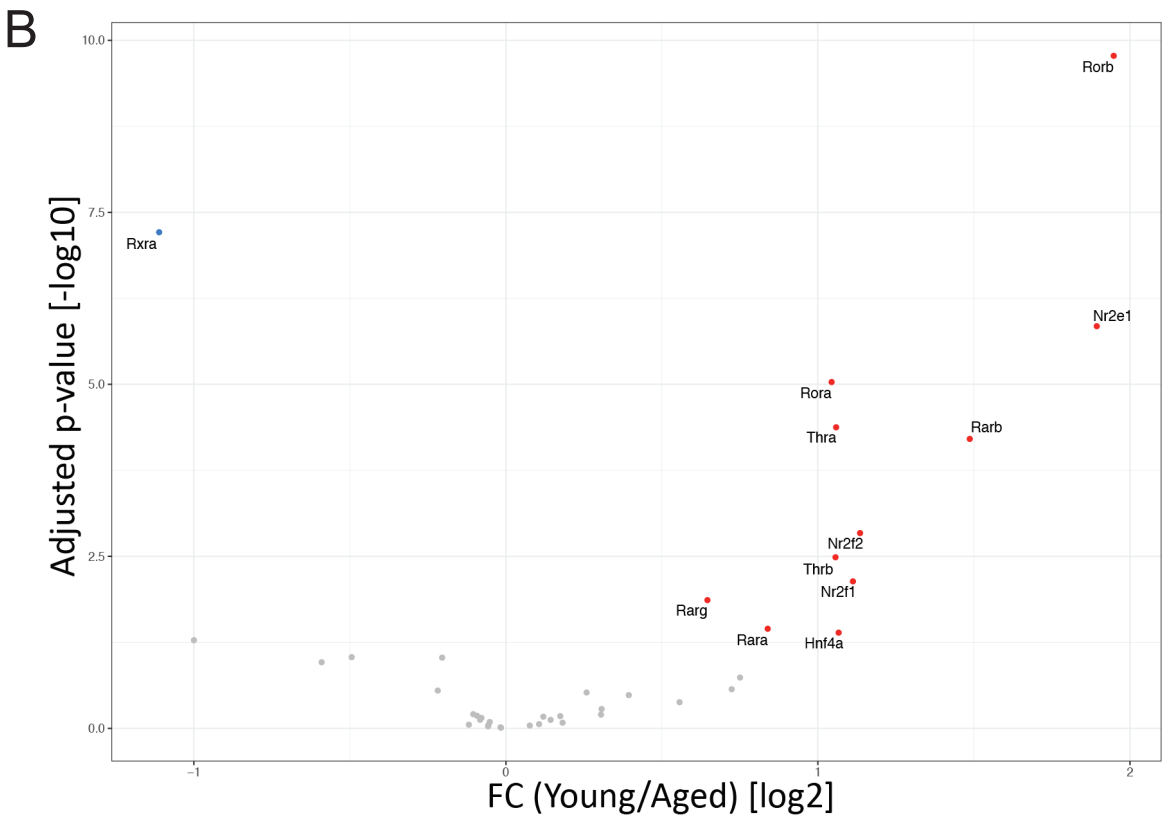
THRα
Nurr1
THRβ
Intensity of NR/actin
Neonate
3
10
18+
Age (months)


B
Adjusted p-value [-log10]
FC (Young/Aged) [log2]
Rorb
Rxra
Nr2e1
Rora
Thra
Rarb
Nr2f2
Thrb
Nr2f1
Rarg
Rara
Hnf4a

C

Gene name	Protein name
Hnf4a	HNF4α
Nr2e1	TLX
Nr2f1	COUP-TF 1
Nr2f2	COUP-TF 2
Rara	RARα
Rarb	RARβ
Rarg	RARγ
Rora	RORα
Rorb	RORβ
Rxra	RXRα
Thra	THRα
Thrb	THRβ

Fig. 3.21 Changes in NR protein and RNA levels in ageing OPCs. Rat primary OPCs were acutely isolated using MACS from rats of four different ages: neonates, three, ten and over 18 months of age. They were lysed and used for western blot to assess the protein levels of a variety of NRs here reported. (A) To assess changes at the protein level western blot analysis for a series of NRs was carried out on primary OPCs MACS isolated from rats of different ages. VDR and LXRα showed no significant differences in the amount of protein expressed at different ages. All data was analysed using ordinary one-way ANOVA, biological n=3. Analysis for the other NRs was not possible as less than three biological replicates were collected. Nevertheless, it is clear that the majority of cases tend to show a decrease in the level of expression of NRs with increasing age. (B) This is supported by the unpublished RNA-seq analysis comparing young and aged rat primary OPCs isolated via MACS. This RNA-seq was carried out and presented with permission from Roey Baror, a former member of the Franklin laboratory. The majority of NRs detected by RNA-seq fall to the right side of the volcano plot, showing that they are expressed at lower levels in aged OPCs compare to young. On the left side of the volcano plot we find RXRα, which is expressed at higher levels in aged OPCs compared to young. (C) Table illustrating the NR gene names on the volcano plot and the corresponding NR protein names.

vasculature with their young counterparts (Ruckh et al., 2012). Indeed, the decrease in NR ligands with ageing can occur for a variety of reasons. However, a decrease in expression of the enzymes involved in the production of such ligands, such as CYP enzymes, has also been reported with increasing age (Wauthier et al., 2007). Unpublished proteomics data from the Franklin laboratory aimed at comparing young and old OPCs has shown ALDH1A1, the enzyme involved in endogenous 9cRA production, to significantly decrease in aged OPCs. Lack of ligands could therefore be a salient aspect of impaired NR signalling in ageing. This would resemble either the charcoal treated or serum-free cultures I have reported in Figure 3.7, where the deficiency of multiple ligands in the environment could lead to a lack of signals and an inability of OPCs to respond appropriately to their environment. As a consequence of this, the regulation of NR target genes would be impaired. Cell cycle regulators such as p27 and CDKs, which are RXR targets in OPCs, have been shown to act aberrantly in a variety of aged cell types (Tamir and Miller, 1999; Janzen et al., 2006; Hershman et al., 2015). It would therefore not be surprising if this combination of events were to lead to an alteration in cell cycle dynamics and potentially impair both proliferation and differentiation of progenitor cells. Furthermore, the above hypothesis would support the observations reported by Huang et al. (2010a), whereby the *in vivo* treatment of aged rats with 9cRA accelerated remyelination. In a scenario where a ligand such as 9cRA may be lacking, its restoration allowed for a gain of function that is typically observed spontaneously in young adults.

Although so far one can only speculate on hypothetical ways in which NR pathways may be impaired in ageing or may contribute to ageing themselves, I believe that the study of NRs in the context of stem cell ageing to be of fundamental importance. Not only because the ageing field has an interest in studying chromatin alterations observed in the ageing phenotype for which NRs may be the bridging molecules, but also because changes in the expression of NRs and alterations in the production of NR ligands have been widely reported in the ageing context. There is a growing interest in studying small lipophilic molecules and their receptors as the field of molecular biology is becoming aware of their importance in the fundamental functions of cellular life.

Chapter 4

Final discussion

4.1 RXR activation facilitates differentiation by influencing OPC proliferation: a new hypothesis

Remyelination is an endogenous regenerative process whereby entire myelin sheaths are restored to demyelinated axons. This reinstates saltatory conduction, resolving functional deficits that would otherwise arise due to the conduction block caused by demyelination. This is a highly efficient process in the young adult CNS (Franklin and ffrench Constant, 2017). However, in a disease setting and with increasing age remyelination is less efficient, resulting in axon degeneration and clinical decline. The decrease in remyelination efficiency has been mainly attributed to a failure in the ability of OPCs to differentiate (Woodruff et al., 2004). Consequently, the development of remyelination therapies has focused on finding methods to enhance OPC differentiation. This involves two strategies:

1. overcoming negative regulators of OPC differentiation, such as efficient clearance of myelin debris (Natrajan et al., 2015);

2. enhancing the differentiation potential of OPCs by targeting positive regulators of differentiation, such as via the drug-targeted activation of RXR (Huang et al., 2010a).

The latter strategy showed great potential in the context of ageing, as *in vivo* administration of the RXR agonist 9cRA accelerated remyelination in aged rats compared to the saline treated controls (Huang et al., 2010a). Ageing adult stem cells are subject to a functional decline due to both intrinsic and extrinsic changes in their microenvironment (López-Otín et al., 2013). As a consequence, the efficiency of all regenerative processes declines with ageing. *In vitro* assays aimed at testing agents acting on molecular targets selected for the enhancement of remyelination usually use cells sourced from neonatal animals or iPSCs, as they are easier to

obtain and culture than aged stem cells, as done by Mei et al. (2014) and Najm et al. (2015). However, the use of young organisms for testing processes affected in old age can often be misleading due to the altered biology of stem cells, as these may not respond in the same way as their young counterparts. Therefore, the observations reported by Huang et al. (2010a) have been crucial in taking forward RXR as a candidate for remyelination therapies. The authors propose that the accelerated effect observed in ageing is because RXR activation, specifically RXRγ based on their microarray data, promotes OPC differentiation as shown by their *in vitro* assays. However, the molecular mechanism by which RXRγ activation regulates OPC differentiation remains unknown.

In my thesis, I addressed this question by using ChIP-seq in order to find which genes RXRγ controls in primary rat OPCs. With this experiment I showed that RXRγ binds in the proximity of genes involved in stem cell regulation and proliferation, and to a lesser extent to genes specific to oligodendrocyte maturation such as *Mobp* and *Enpp6*. These results are further supported by the *in vitro* treatment of MACS isolated OPCs with 9cRA, which resulted in a decrease in OPC proliferation but no difference in the percentage of differentiated progenitors. This observation suggests that unlike what has been previously hypothesised, RXRγ does not directly regulate OPC differentiation. Previous interpretations were based on the use of mixed glia isolated OPCs and, as explained in Section 3.2.3, this exposes progenitors for 10 days to serum, a solution rich in numerous NR ligands. Mixed glia isolation also exposes OPCs to other cell types such as astrocytes and microglia, which could release substances able to promote OPC differentiation, as it has been shown that monocyte conditioned media is able to promote OPC differentiation *in vitro* (Miron et al., 2013). This isolation method can therefore be misleading when used to study NRs for a number of reasons, including the promiscuous binding of RXR where its heterodimers can be activated by a variety of ligands present in the serum, the cross-talk between the signalling pathways of NRs belonging to different classes, and due to the non-linear 'one ligand to one receptor' relationship described in the Introduction (Chapter 1). Therefore, the advantage of MACS isolation is the absence of serum from the cultures, allowing for the observation of the true effect of activating RXR exclusively, without the impact of other ligands.

One of the most interesting RXRγ targets resulting from the ChIP-seq analysis is p27. The canonical role of p27 is the inhibition of cyclin/CDK complexes involved in cell cycle regulation. Consequently, p27 is a cell-cycle regulated protein, whereby in the presence of conditions favourable to proliferation, p27 levels are low, allowing G1-S transition. Instead, growth inhibitory signals cause p27 levels to increase and via the inhibition of various cyclin/CDK complexes, an accumulation of cells in the G1/G0 stage is observed (Agrawal et al., 1996; Zhang et al., 2000). The importance of p27 in cell cycle regulation becomes apparent

in transgenic mice lacking p27, which display an increase in overall size and organ hyperplasia attributed to an increase in cellular proliferation, as well as showing a predisposition to tumour formation (Fero et al., 1996, 1998). Conversely, overexpression of p27 arrests the cell in the G1 phase of the cell cycle (Goukassian et al., 2001; Sharma et al., 2011). The link between cell fate and cell cycle has been extensively studied in the stem cell field. Stem cells present a rapid cell cycle compared to somatic cells, a feature mainly attributed to the length of G1. Indeed, a correlation between the length of G1 and the pluripotent capacity of cells is observed; naïve cells present a shortened G1 lacking the G1 checkpoint regulation, and primed pluripotency, whether induced or spontaneous, leads to G1 lengthening (Coronado et al., 2013). This relationship is causal as shown by the analysis of FACS-sorted mouse ESCs (mESC) using the FUCCI reporter. FACS-isolated mESCs in the G1 phase present a higher percentage of differentiated colonies compared to mESCs in the S and G2/M phases, under both untreated conditions and after exposure to pro-differentiation signals (Coronado et al., 2013). The above has also been reported in human ESCs, suggesting that cells are more sensitive to commit and differentiate when in the G1 phase (Sela et al., 2012; Calder et al., 2013; Pauklin and Vallier, 2014). During G1, the cell undergoes a period of biosynthesis and chromatin reorganisation necessary to undertake the following round of replication. However, it is also the stage where decision in cell fate is made as the cell is more susceptible to regulatory signals, including pro-differentiating factors (Sela et al., 2012). Therefore a longer G1 holds the cell in a decision-making state, raising the probability of encountering pro-differentiation factors and giving time for lineage-specific transcription factors to bind relevant targets, thereby enhancing the probability of cell cycle exit and differentiation.

Based on the above, I present an alternative hypothesis to what has been previously proposed (illustrated in Figure 4.1). In this model RXR activation, specifically RXRγ, leads to an increase in p27 transcription which consequently influences the OPC cell cycle by lengthening the G1 phase and increasing the likelihood of cell cycle exit. However, the presence of other NR ligands is required to induce terminal differentiation. Potential ligands are T3 and vitamin D, activators of THR and VDR, as these have been reported to promote OPC differentiation (Barres et al., 1994; de la Fuente et al., 2015). In this model, for example, the simultaneous effect of 9cRA and T3 treatment should induce a higher percentage of differentiation than T3 alone, a result which has been observed in MACS isolated OPCs as reported in Figure 3.7C of the Results Section. Thus RXRγ aids differentiation, but is not essential to it, as supported by the work carried out by Huang et al. (2010a), where RXR activation accelerated remyelination but RXRγ absence only delayed remyelination without impairing it. Instead, it would be interesting to assess the proliferation, p27 levels and cell cycle dynamics of OPCs derived form RXRγ *null* mice.

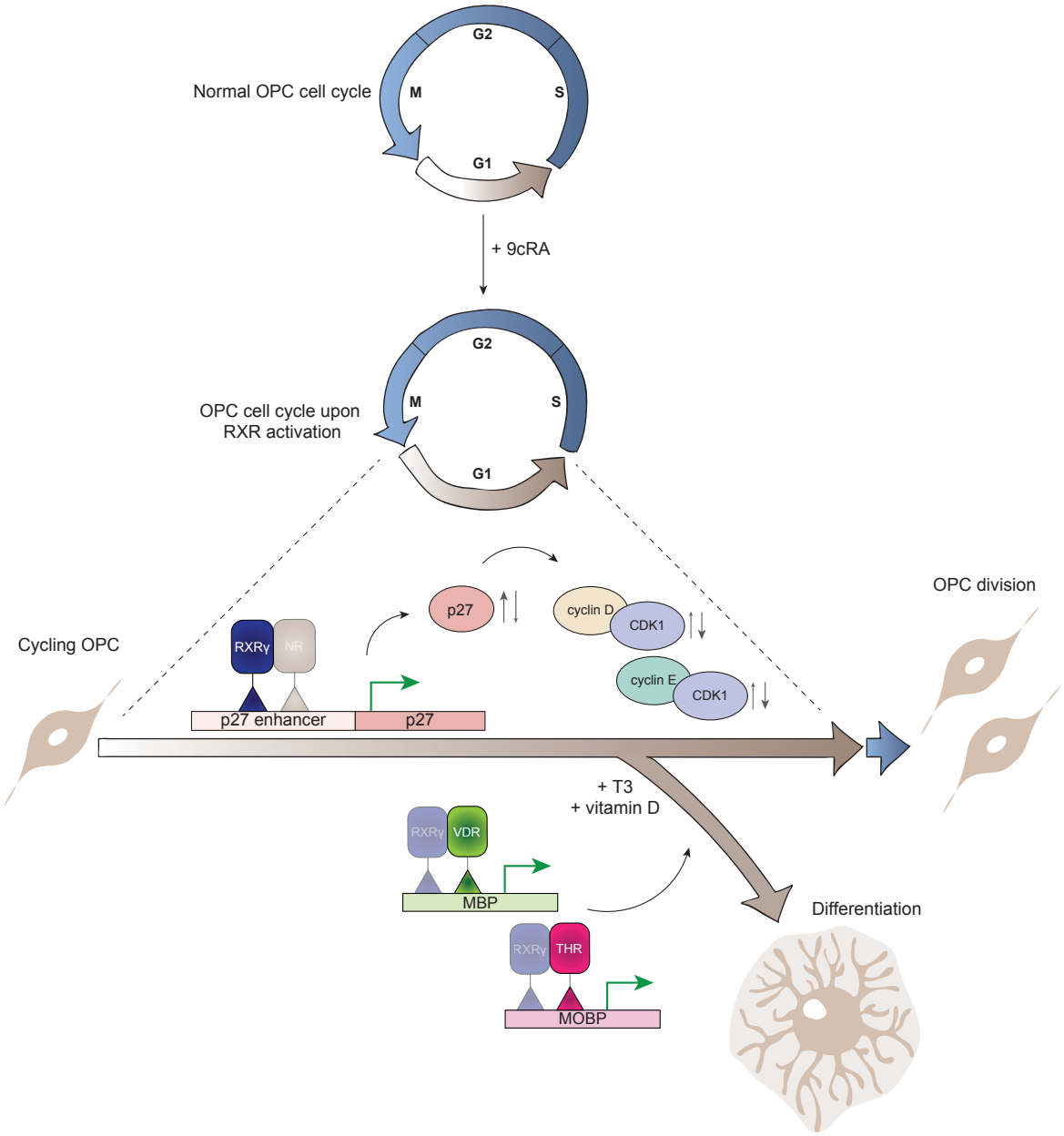


Fig. 4.1 RXR activation facilitates differentiation by influencing OPC proliferation: a new model. Under normal conditions OPCs present a normal cell cycle. In the presence of an RXR ligand such as 9cRA, RXRγ is activated and binds to a p27 enhancer, leading to an increase in transcription of p27. Whether RXRγ binds as a monomer or a dimer still needs to be understood. The p27 increase consequently decreases the activity of cyclin/CDK complexes involved in G1-S transition such as cyclins D and E. This influences the OPC cell cycle by lengthening the G1 phase, thereby maintaining the OPC in the decision-making stage and increasing the likelihood of cell cycle exit. If no other pro-differentiation signals are present, the OPC will likely re-enter S phase for another cycle round. However, in the presence of pro-differentiation factors, the OPC will undergo differentiation. Potential factors are T3 and vitamin D, agonists of THR and VDR which have been shown to promote OPC differentiation. They also could associate as monomers, homodimers or RXR heterodimers. Adapted from Pauklin and Vallier (2014).

As highlighted in Section 3.4, retinoids and rexinoids have been of great interest to the development of cancer therapies due to the inhibitory effect they exert on proliferation. Mechanistically, the effect of RXR activation on increased p27, decreased proliferation and G1 arrest has been observed in cancer cell lines (Wu et al., 2006; Chen et al., 2016; Chou et al., 2018). The use of RXR agonists such as bexarotene and 9-*cis* UAB30 both increase p27 levels in different cancerous cell lines, leading to their arrest in the G1 phase (Chou et al., 2018). However, unlike the experiments reported in this thesis, the authors do not observe an increase in p27 mRNA, therefore they propose that the increase in p27 protein is due to the inhibition of its degradation. In other cancerous cell lines effects on decreased proliferation and G1 arrest after rexinoid treatment have also been reported. However, no changes in p27 levels were observed despite the above (Wu et al., 2006). The opposite has also been reported, whereby RXR inhibition leads to G1 arrest of primary adipocytes derived from a rat model for obesity and type II diabetes, as well as in cell lines of preadipocytes and mesangial cells (Nakatsuka et al., 2012). Despite the contradicting literature, all of the results reported above have been mainly observed in cell lines or in animal models used for diseases. These cells and animals do not present a normal physiological state as a baseline, therefore there is the potential for the results to be dependent on the specific condition of such models. Furthermore cell-specific effects of RXR could contribute to the differences observed by studies reported above. Further research involving primary cells and the specific cell-type of interest are necessary in order to elucidate the exact mechanism by which RXR activation influences the G1 phase of the OPC cell cycle.

4.2 RXR activation and G1 lengthening in the ageing OPC

The experiments reported in this thesis have been carried out on primary neonatal rat OPCs. However, as stated above, the use of ageing cells is fundamental to the development of treatments aimed at targeting processes impaired with ageing. Whilst the the model described is yet to be validated in neonatal OPCs, an interesting and valid question is how would the model proposed work in ageing OPCs which have been reported to have a longer cell cycle *in vivo* compared to their younger counterparts (Lasiene et al., 2009; Young et al., 2013). This is believed to be due to an increase in G1 lengthening, as this feature is observed in numerous, albeit not all, ageing progenitor cells (Geha et al., 2010; Daynac et al., 2014; Kowalczyk et al., 2015). If ageing does lead to a slower progression through G1, it would be relevant to verify which G1 sub-phase is specifically affected in both ageing and via RXR activation.

G1 is split into two sub-phases, early G1 and late G1, which are divided by the restriction point (R point). At the R point, the progenitor commits to another round of cycling and

is characterised by the phosphorylation of retinoblastoma protein, which releases the transcription factor E2F, responsible for the transcription of genes involved in S phase transition. Early G1 is therefore the sub-phase where the progenitor is most susceptible to signals determining cell fate (Chen et al., 2015). RXR activation should therefore induce the lengthening of early G1 in agreement with the model presented above. Adult human OPCs have been proposed to have a lengthened early G1. However, only five subjects were studied in an age range of 25–66 years of age (Geha et al., 2010). Thus, this study lacked a representative number of subjects per age as well as subjects of older ages, and borders on what is considered to be the age milestone for acquiring permanent disability in MS (Confavreux and Vukusic, 2006). The impairment of aged OPCs may therefore reside in a lengthened late G1, whereby the majority of progenitors are in G1 but cannot proceed to S phase due to impaired cell cycle, and at the same time cannot differentiate as they are past the R point. In this scenario, lengthening early G1 would still be beneficial, as this would delay reaching the R point and enhance the responsiveness of aged OPCs to differentiation signals. To determine the validity of this model in aged OPCs, it is important to understand which G1 sub-phase is affected by RXR and which is altered in the ageing OPC cell cycle.

However, an extended G1 phase as a consequence of ageing still needs to be confirmed in OPCs. Furthermore, despite the *in vivo* observation that aged OPCs in both humans and rodents present a longer cell cycle than their younger counterparts, *in vitro* assessment of young and old MACS isolated OPCs does not present any statistically significant differences upon assessment of EdU incorporation. These results are not presented in this thesis but have also been observed by other members of the lab. This hints at a similar cell cycle length between young and old OPCs, whilst neonatal OPCs have a much shorter cell cycle length. Therefore the ageing phenotype of OPCs resides in the inability to respond to pro-differentiation signals efficiently and quickly. However, projects in the lab have shown that aged OPCs can be manipulated to respond more efficiently to differentiation signals, for example by altering their nutrient sensing pathways and by altering the mechanical stiffness of their surrounding microenvironment. Lengthening early G1 may indeed give the slow-responding ageing OPCs more time to not only detect differentiation signals, but also to commit to the fate imposed by the signals detected by allowing transcription factors to transcribe the genes required for maturation and differentiation.

4.3 Is targeting RXR and its partners a valid strategy for remyelination therapies in MS?

The accelerated remyelination observed by Huang et al. (2010a) after RXR activation *in vivo* was initially attributed to an effect of RXRγ on the differentiation capacity of OPCs. In this thesis I propose that unlike what was previously suggested, RXR does not directly influence differentiation. Instead, RXR activation may sensitise the cell to pro-differentiation signals in its microenvironment, thereby aiding the differentiation process. In the light of what has been presented and what is known, is targeting RXR and its partners a valid strategy for remyelination therapies?

Both RXR and its partners are important players in the development and regeneration of a variety of organs including liver, bone and skin (Scheven and Hamilton, 1990; Togari et al., 1991; Warner et al., 2008; Hellemans et al., 1999; Bruck et al., 2009). In these organs NRs have been involved in numerous biological processes important to regeneration, including inflammation and angiogenesis, as reviewed by Jin et al. (2015); Rieger et al. (2015); Rudraiah et al. (2016). Consequently, NR activation in other cell types can be beneficial to the regenerative process. Indeed, the activation of RXR in innate immune cells resulted in an increase in their phagocytic capability despite their old age, and mice lacking RXRα presented reduced phagocytic activity and a delay in remyelination (Natrajan et al., 2015). The positive effect of RXR activation has also been reported in murine models for MS. Indeed, in the EAE model, RXRγ activation via agonists such as 9cRA and IRX4204 resulted in the amelioration of disease severity and prevented disease progression compared to the control group (Diab et al., 2004; Takeuchi et al., 2013; Chandraratna et al., 2016). This is because rexinoids exert a strong immunomodulation by decreasing the proliferative potential of $CD4^+$ T cells that produce pro-inflammatory cytokines and increases the expression of CTLA-4, involved in the down-regulation of the immune response (Takeuchi et al., 2013; Chandraratna et al., 2016). Hence targeting RXR can modulate both the innate and adaptive immune system, as well as OPCs, thereby attenuating the inflammatory response, reducing the extent of damage and aiding the regenerative process. Furthermore, RXRγ has been shown to mediate the action of mTORC1 in Schwann cells, by transcribing sterol regulatory element-binding protein 1c (SREBP1c) through which mTORC1 regulates lipid biosynthesis which, if compromised, leads to Schwann cell hypomyelination (Norrmén et al., 2014). This could also be relevant to OPCs, as studies in the lab have shown that calorie restriction, a paradigm that affects mTORC1, restores regenerative function in aged animals by increasing the differentiation efficiency of aged OPCs. This could be one of the ways in which RXRγ sensitises the cell to pro-differentiation signals.

All the above observations suggest that targeting RXR would have clear benefits in the context of a chronic demyelinating disorder such as MS, as it could hypothetically decrease the severity of the immune attacks and aid the efficiency of remyelination, thereby limiting the damage and delaying accumulation of disability. In humans, studies have pointed towards a link between low amounts of NR ligands and the risk of developing MS. For example, both vitamin D and RA serum levels have been used as predictors of MS, as their levels are lower in MS patients compared to controls (Royal et al., 2002; Ascherio et al., 2014). Based on these epidemiological observations MS patients were administered vitamin D and retinyl palmitate with the hope of ameliorating the disease. However, no improvements were observed on clinical scores and relapse rates (Bitarafan et al., 2013; Jafarirad et al., 2013). Lower levels of PPARγ in the cerebral spinal fluid and higher levels of anti-thyroid autoantibodies have also been found in MS patients compared to healthy controls. However, attempts at using these features as biomarkers for the risk of developing MS and as predictors of relapses failed due to the lack of a clear relationship (Szalardy et al., 2013; Fallahi et al., 2017). At the same time, larger clinical studies with a longer administration course of vitamin A presented significant improvements in the performance of the MS functional composite test, which measures the progression of disability in both cognitive and upper limb function (Bitarafan et al., 2015). Furthermore, a phase II trial assessing the use of agonists targeting class III NRs, such as using a combination of estriol and glatiramer acetate, reduced the rate of relapses (Voskuhl et al., 2016).

Based on the above, I believe that RXR and other NRs have great potential in the development of remyelination therapies. The most interesting aspect of RXR and NR biology is their ability to influence both proliferation and differentiation. This is especially interesting in the context of MS, as this disorder presents heterogeneous lesions, where approximately 30% of the lesions lack OPCs whilst the remaining are abundant in OPCs but remyelination fails due to a failure in their differentiation (Lucchinetti et al., 1999; Boyd et al., 2013). This suggests that the development of a therapy aimed at targeting either OPC proliferation or OPC differentiation would be inefficient in a large proportion of the lesions presented. The use of NR ligands opens new avenues for the development of patient tailored therapies which could target either or both processes, according to the need of the individual. However, a careful characterisation of their signalling pathway and an appropriate clinical trial design are necessary in order to properly predict and assess the true outcome of their manipulation in the disease context.

4.4 The complexity of NR signalling and clinical trial design

In recent years there has been a considerable interest in the use of NRs as therapeutic targets for both MS and remyelination, as shown by the current efforts of testing agonists for GCR, THR, VDR and RXR (de la Fuente et al., 2015; Najm et al., 2015; Zhang et al., 2016b; Plemel et al., 2017). This is due the numerous appealing principles of NR biology, including:

- their evolutionary conservation across organisms which should make the use of animal models representative and translatable to humans;
- they are ligand-induced and therefore easily targetable with small molecules;
- they present a direct bridge for the manipulation of gene transcription;
- they signal through a common network at which we can intervene at various points;
- drugs targeting NRs are already FDA approved for cancer treatments, which should facilitate their approval and production process.

Despite their biology pointing towards success in remyelination in animal models as well as in their translation to the clinic, clinical trials still fail. The reason lies in their biological and molecular complexity, the current animal models used to study remyelination in MS as well as our lack of knowledge on how NRs function, all of which hinder the design of proficient clinical trials. Due to the pleiotropic effects exhibited by NRs, the use of rexinoids presents potent side effects. This leads to a high drop out rate and not enough patients to conclude clinical trials. Furthermore, if the drugs were to be approved despite the strong side effects, the therapy could lead to the requirement of a complex drug regime to counteract these issues. Complex drug regimes are problematic to both patients and medics, as they are expensive and cause issues of non-adherence and adverse drug reactions (Golchin et al., 2015).

Clinical trials are also ineffective due to the lack of a single appropriate animal model for studying MS, a topic reviewed by Franklin and ffrench Constant (2017). In particular, we lack a model for the progressive phase of the disease, which is currently untreatable by the currently available disease modifying drugs. In order to assess remyelination, toxin models of demyelination are used. However, these lack the important interaction between the detrimental inflammation observed in MS and the regenerative process. Furthermore, most of the NR studies carried out use the cuprizone model for which an attentive experimental design is required, as explained in the Introduction (Chapter 1). NR ligands have been studied much more in the context of EAE. However, EAE does not recapitulate the chronic

aspect of MS and makes it complicated to distinguish the true effect a molecule may have on remyelination due to the strong inflammatory reaction. Therefore these studies concentrate on the effect NRs have on the immune system rather than on remyelination itself. As a consequence, the use of either model comes with the risk of identifying a promising target which may ultimately fail in clinical trials.

The ultimate reason for weak clinical trial outcomes lies in the lack of knowledge of how the NR system functions and signals. This can prompt researchers to ask the wrong questions and draw the wrong conclusions from studies. The NR field of research is relatively young, as it has been approximately 16 years since the discovery of multiple NR isoforms and the realisation that they function via association to co-regulators that allow for chromatin remodelling (Tata, 2002). The above two discoveries are central to the modern understanding of how NRs signal, but have been extremely recent. The above begs the question of how much more is still to be understood and discovered. Therefore, a better *in vitro* and *in vivo* characterisation of both NR structure and function is required before venturing into human clinical trials.

In this thesis I characterised the different levels of RXR signalling in primary OPCs. Although the results require further validation, I believe this characterisation to be fundamental in contributing to the overall picture of NR signalling, as this knowledge will aid the reduction of undesired side effects that would arise from using NRs as a therapy for remyelination. By understanding which RXR heterodimer complex is involved in the transcriptional regulation of the gene necessary for the desired outcome, one can try to target the complex via the NR partner or the co-regulators instead of RXR itself. This would limit the side effects that arise from activating all RXR heterodimers in the cell of interest, as well as in other cell types. For example, I have shown that the RXRγ principally binds to LXRβ at the OPC stage of the lineage. Despite not knowing the true biological significance of this, it hints at an important role for RXRγ-LXRβ in OPCs. Whether RXRγ-LXRβ is important in regulating the OPC cell cycle is still unknown, but its involvement in the regulation of cholesterol and fatty acid metabolism has been extensively described (Courtney and Landreth, 2016b). If activating this heterodimer aids oligodendrocyte formation by influencing cholesterol metabolism to meet differentiation demands, then this could be a valid target for remyelination therapies. At this point, with the use of ligands specific to LXRβ, one should achieve the transcription of genes specific to cholesterol metabolism, thereby sparing the effect that RXRγ activation would have on the cell cycle or other biological processes that need to remain unaffected. This is an oversimplified example, but the raw ChIP-seq data reveals that RXRγ binds proximal to about half of the total protein coding genes of the genome, suggesting its importance in influencing almost all housekeeping cellular processes (Figure 3.15B). Due to this, tar-

geting RXRγ exclusively may not be the optimal strategy going forward in the development of a therapy. It is therefore important to select the biological process that needs to be targeted, whether this is cell cycle regulation, migration, lipid metabolism, differentiation and so on, in order to fully understand which partner and co-regulators are responsible, and to consequently produce patient specific therapies. Taken together, I believe that a better characterisation of the complex and interconnected signalling of RXRγ heterodimers is required, as this knowledge is essential in choosing the most appropriate target and suitable ligands, allowing optimal clinical trial design.

4.5 Directions for future research

Throughout this thesis I have demonstrated that much more work is required in order to validate the observations and new hypotheses presented, as well as to take forward the knowledge of how RXRγ regulates oligodendrocyte lineage cell function.

Despite the ChIP-seq results being extremely interesting and informative on RXRγ binding dynamics and genomic preferences, additional experiments are required to determine whether RXRγ regulates genes involved in OPC differentiation and oligodendrocyte maturation. As well as ChIP-seq, it would be interesting to carry out RNA-seq of 9cRA and vehicle control treated OPCs. Unlike ChIP-seq, which shows the binding location of transcription factors, RNA-seq is much more informative with regards to the actual change in gene expression as a consequence of a treatment or the regulation of a transcription factor of interest. The outcome of the experiment would be the generation of a transcriptional profile governed upon RXRγ activation in OPCs, leading to a more complete picture of the functional consequences of RXRγ activation. This is especially important following the results generated via ChIP-seq, which show that upon 9cRA treatment RXRγ associates to regulatory regions of the genome and consequently could be regulating any protein coding gene in the genome. As well as answering the original hypothesis, this experiment would lead to the generation of candidates presenting transcriptional changes that could be used as a direct functional read out for any future experiment assessing RXRγ activity in the oligodendrocyte lineage.

In Section 3.2.4 I have shown that RXRγ does not physically switch binding partners as OPCs progress throughout the lineage. The aim was to determine whether OPC differentiation and oligodendrocyte maturation are regulated by specific RXRγ heterodimers, and whether certain binding partners are more relevant than others at different stages of the lineage. The use of Duolink® to determine the above has given insight on the association dynamics between RXRγ and its partners. However, physical association may not directly

translate to functional relevance. Therefore, determining the functional relevance of these RXRγ heterodimers when it comes to OPC proliferation, differentiation and oligodendrocyte maturation is fundamental when assessing predominant binding partners at different stages of the lineage. To answer the above question, simple *in vitro* studies can be carried out whereby OPCs are treated with 9cRA together with a partner antagonist, and if the 9cRA effect is abrogated compared to the 9cRA treatment alone, then it can be proposed that the specific effect being observed is elicited by that specific heterodimer. If this were to be carried out for proliferation, differentiation and maturation, or using changes in transcripts obtained via the previously mentioned RNA-seq, then we would have new insights into which RXR heterodimers are important in eliciting a specific effect or result in a certain outcome.

An interesting observation resulting from the ChIP-seq experiment is that RXRγ largely associates to intergenic and intronic genomic areas known to contain numerous regulatory elements. RXRγ rarely binds to promoter regions or in proximity of transcription start sites of protein coding genes. This observation has led me to hypothesise that RXRγ does not directly regulate transcription of target genes. Instead, it purely determines the likelihood or probability of a gene being transcribed or an effect occurring upon its activation. In this scenario, RXRγ would act as a factor priming the OPC for a response by regulating the state of the chromatin, but other signals and factors would ultimately be required to execute the change in gene transcription. Therefore, chromatin modifications due to RXRγ activation or repression would tip the balance in favour of certain transcriptional profiles, thereby facilitating certain cellular and physiological outcomes. It would be interesting to test this hypothesis using a paradigm where cells are no longer responsive to conventional OPC differentiation signals, such as the MACS isolated aged OPC. By using aged OPCs, one can carry out treatment combinations such as 9cRA and T3, calcitriol or ATRA, and determine whether priming cells via RXR activation can restore the response to the original NR ligands. This can be carried out with a variety of compounds as well as conditioned media from other cell types, known to promote OPC differentiation. Although it has been shown that aged OPCs do not respond to 9cRA treatment *in vitro* as mixed glia cells do, it is important to consider that these cells were MACS isolated and cultured in serum-free media. This could result in the same outcome observed when treating MACS sorted neonatal OPCs with 9cRA in serum-free conditions, a result that could partly be due to the paradigm being used. Overall, it would be interesting to understand if RXR activation can indeed aid in priming OPCs to differentiate when used in a paradigm known to not respond efficiently to differentiation signals.

All of the above experiments have the additional advantage of avoiding the use of RXRγ antibodies. Following from the mass spectrometry experiments it is clear that NR antibody validations are fundamental in order to fully trust experimental outcomes. Such antibodies can be validated by carrying out a simple western blot on cells derived from an RXRγ knockout mouse, or on cell lines in which *in vitro* RXRγ knockout using CRISPR was carried out. Furthermore, if the effect of RXRγ is observed *in vivo*, in the presence of other cell types or conditioned media, then the MACS paradigm may be too simplistic and result in the abrogation of the RXRγ effect. Due to the ubiquitous expression of RXR, its ligands are able to affect numerous different cell types including neurones, astrocytes and microglia. Therefore, it is important to use paradigms that also include other cell types, such as co-cultures, *ex vivo* cerebellar slices and *in vivo* assessments in order to validate what is carried out *in vitro* if the ultimate goal is the development of successful remyelination therapies.

Chapter 5

Concluding remarks

In this thesis I have shown that RXRγ signalling does not influence OPC differentiation directly, as 9cRA treatment in serum-free cultures did not induce oligodendrocyte formation. Instead, using a ChIP-seq approach, I have demonstrated that RXRγ influences genes involved in proliferation and cell cycle control. They probably do so via the activation of non-protein coding regulatory regions, a hypothesis which will need to be verified with the use of reporter assays. Further functional assessments aided me in the development of a new hypothesis where RXRγ activation sensitises the OPC to pro-differentiation signals. Based on the targets obtained, such as the cell cycle exit marker p27, I developed a yet to be validated hypothesis where RXRγ activation lengthens the G1 phase of the OPC cell cycle, thereby increasing the likelihood of the cell to detect pro-differentiation signals and fully commit to terminal differentiation. It will be interesting to understand which NR partner(s) form the RXRγ heterodimer, if any, involved in regulating the OPC cell cycle. However, many more genes involved in processes important to the oligodendrocyte lineage resulted from the ChIP-seq experiment. One of these is cholesterol and lipid metabolism, a fundamental aspect of oligodendrocyte formation, which presents an alternative and interesting avenue worth exploring further. The above demonstrates that NRs signal in complex ways with unexpected outcomes, and whilst the use of rexinoids has the potential for the development of remyelination therapies, their use may not suffice in human clinical trials prior to acquiring further knowledge on how these receptors signal.

Discoveries in the NR field have gone hand in hand with the development of new technologies, from ChIP to X-ray crystallography and NMR. Consequently, as new experimental techniques are developed, it will be inevitable for our understanding of NRs to change further, creating an increasingly complicated picture. With this in mind, there is an urgent need for collaboration and data integration of the broader scientific community if we want the use of NRs in remyelination therapies to become a reality.

‘Cominciate col fare ciò che è necessario, poi ciò che è possibile.
E all’improvviso vi sorprenderete a fare l’impossibile.’
- San Francesco d’Assisi

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Appendix A

Product	Provider	Catalogue number
1-850	Calciochem	609315
1a25-Dihydroxyvitamin D3	Sigma Aldrich	D1530
22(S)-Hydroxycholesterol	Sigma Aldrich	H5884
9 *cis* retinoic acid	Sigma Aldrich	R4643
Activated charcoal	Sigma Aldrich	C9157-500g
All-*trans* retinoic acid	Sigma Aldrich	R2625
Apo-transferrin	Sigma Aldrich	T1147-100MG
B27	Gibco Life Tech	17-504-044
BCA assay kit	ThermoFisher	23227
BSA	Gibco Life Tech	15260-037
Bolt 4-12% bis/tris plus Gel	Invitrogen	NW04120BOX
CD2314	Tocris	3824
D-(+)-glucose	Sigma Aldrich	G8644-100ML
DMEM	Gibco Life Tech	41966-029
DMEM-F12	Gibco Life Tech	11039-021
DMSO	Sigma Aldrich	D8418
DNAse I Type IV	Sigma Aldrich	D5025-150KU
Direct-zol RNA MicroPrep Kit	Zymo Research	R2060
Duolink® Click-iT Plus EdU AlexaFluor 488 Kit	ThermoFisher	C10637
Duolink® Detection reagent orange	SigmaAldrich	DUO9007
Duolink® PLA probe anti-mouse PLUS	Sigma Aldrich	DUO9001
Duolink® PLA probe anti-rabbit MINUS	Sigma Aldrich	DUO9005
Duolink® Wash Buffers	Sigma Aldrich	DUO82049
EDTA	Invitrogen	15575-038
EZ-chIP coimmunoprecipitation kit	Millipore	17-371

Ethanol	Sigma Aldrich	32221
Fetal bovine serum	Biosera	8670,9910,3040
Fluoromount G	Southern Biotech	0100-01
Gel purification QIAEX II Kit	Qiagen	20021
H_2O RNase/DNase free	Invitrogen	10977-035
HALT protease & phosphatase inhibitor	ThermoFisher	PN78441
HBSS-/-	Gibco Life Tech	14170-088
HX531	Tocris	3912
Höechst	Sigmal Aldrich	33258
Immobilon FL PVDA transfer membrane	Merck Millipore	IPFL00010
Insulin	Gibco Life Tech	12585-014
LDS Sample Buffer (4X)	Invitrogen	NP0007
LE135	Tocris	2012
MOPS SDS Running Buffer 20X	Invitrogen	NP0001
MS columns	Myltenyi Biotech	130-042-201
Magnet	Myltenyi Biotech	
Methanol	Honeywell	32213
Mouse anti-A2B5 IgM antibody	Millipore	MAB312
Mycozap Plus-PR	Lonza	VZA-2011
N-Acetyl-L-cysteine	Sigma Aldrich	A7250
Normal donkey serum	Sigma Aldrich	D9663
Odyssey Blocking Buffer	Li-Cor	927-50000
PBS 10X (-/-)	Gibco Life Tech	70013-016
Papain	Worthington	3126
Paraformaldehyde	Fisher Scientific	P/0840/53
Penicillin streptomycin	Sigma Aldrich	P4333
Pentoject	Animalcare	XVD 135
Percoll	GE Healthcare	17-0891-01
Phosphate buffered saline 10X	Fisher Bioreagents	BP-39920
Pierce Crosslink IP Kit	ThermoFisher	26147
Pierce IP Lysis buffer	ThermoScientific	87787
Pluronic acid	Gibco Life Tech	24040-032
Poly D-lysine	Sigma Aldrich	P6407
Precision plus protein standard	BioRad	161-0374
Progesterone	Sigma Aldrich	P-8783
Propidium Iodide Flow Cytometry Kit	Abcam	ab139418

Propidium iodide	Invitrogen	P3566
Putrescine	Sigma Aldrich	P5780
Quantitect Reverse Trancription Kit	Qiagen	205310
RXRγ recombinant protein	Novus	NBC1-18416
Rat anti-mouse IgM MicroBeads	Myltenyi Biotech	130-047-301
Recombinant human FGF-basic	Peprotech	100-18B
Recombinant human PDGF-AA	Peprotech	100-13A
Rosiglitazone	Sigma Aldrich	R2408
SYBR Green PCR Master Mix	ThermoFisher	4309155
Sample Reducing Agent (10X)	Invitrogen	NP0004
Sodium pyruvate	Invitrogen	11360-039
Sodium selenite	Sigma Aldrich	S5261
T 0901317	Tocris	2373
T00709	Sigma Aldrich	T8703
Tetraethylthiuram disulfide	Sigma Aldrich	86720
TGS Tris/Glycine/SDS Transfer Buffer 10X	BioRad	161-0772
Triiodothyronine	Sigma Aldrich	T6397
Tris buffered saline 10X	Fisher Bioreagents	BP2471-1
Triton-X 100	Sigma Aldrich	T9284
Trizol	ThermoFisher	15596026
TrypLE Express Enzyme (1X)	ThermoFisher	12604013
Tween	Sigma Aldrich	93773-250G
Western Blot Filter Paper	ThermoFisher	88600
Zombie violet	BioLegend	423113

Table A.1 Table of all the materials used.